



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

|  |           |  |
|--|-----------|--|
| <b>(51) International Patent Classification <sup>5</sup> :</b><br><b>C12Q 1/68, C07H 15/12, 17/00</b>  | <b>A1</b> | <b>(11) International Publication Number:</b> <b>WO 92/17484</b><br><b>(43) International Publication Date:</b> 15 October 1992 (15.10.92)   |
| <b>(21) International Application Number:</b> PCT/US92/02480<br><b>(22) International Filing Date:</b> 26 March 1992 (26.03.92)<br><b>(30) Priority data:</b><br>675,843 27 March 1991 (27.03.91) US<br><b>(71) Applicant:</b> RESEARCH CORPORATION TECHNOLOGIES, INC. [US/US]; 6840 East Broadway Boulevard, Tucson, AZ 85710 (US).<br><b>(72) Inventor:</b> KOOL, Eric, T. ; 39 Brighton Street, Apt. 1, Rochester, NY 14600 (US).<br><b>(74) Agent:</b> SCOTT, Anthony, C.; Scully, Scott, Murphy & Presser, 400 Garden City Plaza, Garden City, NY 11530 (US).   |           | <b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), BR, CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), MC (European patent), NL (European patent), NO, SE (European patent).<br><br><b>Published</b><br><i>With international search report.</i> |
| <b>(54) Title:</b> SINGLE-STRANDED CIRCULAR OLIGONUCLEOTIDES<br><br><b>(57) Abstract</b><br><br>The present invention provides single-stranded circular oligonucleotides each with a parallel binding (P) domain and an anti-parallel binding (AP) domain separated from each other by loop domains. Each P and AP domain has sufficient complementarity to bind to one strand of a defined nucleic acid target wherein the P domain binds in a parallel manner to the target and the AP domain binds in an anti-parallel manner to the target. Moreover, the present single-stranded circular oligonucleotides can bind to both single-stranded and double-stranded target nucleic acids. The present invention also provides methods of using these oligonucleotides as well as pharmaceutical compositions containing these oligonucleotides. |           |  |

**PCT/US92/02480**

L. YUAN

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

|    |                          |    |  |    |                          |
|----|--------------------------|----|--|----|--------------------------|
| AT | Austria                  | ES | Spain                                    | MG | Madagascar               |
| AU | Australia                | FI | Finland                                  | ML | Mali                     |
| BB | Barbados                 | FR | France                                   | MN | Mongolia                 |
| BE | Belgium                  | GA | Gabon                                    | MR | Mauritania               |
| BF | Burkina Faso             | GB | United Kingdom                           | MW | Malawi                   |
| BG | Bulgaria                 | GN | Guinea                                   | NL | Netherlands              |
| BJ | Benin                    | GR | Greece                                   | NO | Norway                   |
| BR | Brazil                   | HU | Hungary                                  | PL | Poland                   |
| CA | Canada                   | IT | Italy                                    | RO | Romania                  |
| CF | Central African Republic | JP | Japan                                    | RU | Russian Federation       |
| CG | Congo                    | KP | Democratic People's Republic<br>of Korea | SD | Sudan                    |
| CH | Switzerland              | KR | Republic of Korea                        | SE | Sweden                   |
| CI | Côte d'Ivoire            | LI | Liechtenstein                            | SN | Senegal                  |
| CM | Cameroon                 | LK | Sri Lanka                                | SU | Soviet Union             |
| CS | Czechoslovakia           | LU | Luxembourg                               | TD | Chad                     |
| DE | Germany                  | MC | Monaco                                   | TG | Togo                     |
| DK | Denmark                  |    |  | US | United States of America |

-1-

1            SINGLE-STRANDED CIRCULAR OLIGONUCLEOTIDES

The present application is a continuation-in-part of copending U.S. Serial No. 675,843 filed March 5 27, 1991. Moreover, the subject matter of the present application relates to subject matter contained in Disclosure Document number 234,794 received by the United States Patent and Trademark Office on September 5, 1989.

10            This invention was made with United States government support under grant number GM-46625 awarded by the National Institutes of Health. The United States government has certain rights in the invention.

15 FIELD OF THE INVENTION:

The present invention provides single-stranded circular oligonucleotides capable of binding to a target DNA or RNA and thereby regulating DNA replication, RNA transcription, protein translation, and other processes  
20 involving nucleic acid templates. Furthermore, circular oligonucleotides can be labeled for use as probes to detect or isolate a target nucleic acid. Circular oligonucleotides can also displace one strand of a duplex nucleic acid without prior denaturation of the  
25 duplex. Moreover, circular oligonucleotides are resistant to exonucleases and bind to a target with higher selectivity and affinity than do linear oligonucleotides.

30 BACKGROUND OF THE INVENTION:

An oligonucleotide binds to a target nucleic acid by forming hydrogen bonds between bases in the

35



1 target and the oligonucleotide. Common B DNA has  
conventional adenine-thymine (A-T) and guanine-cytosine  
(G-C) Watson and Crick base pairs with two and three  
hydrogen bonds, respectively. Conventional  
5 hybridization technology is based upon the capability of  
sequence-specific DNA or RNA probes to bind to a target  
nucleic acid via Watson-Crick hydrogen bonds. However,  
other types of hydrogen bonding patterns are known  
wherein some atoms of a base which are not involved in  
10 Watson-Crick base pairing can form hydrogen bonds to  
another nucleotide. For example, thymine (T) can bind  
to an A-T Watson-Crick base pair via hydrogen bonds to  
the adenine, thereby forming a T-AT base triad.  
Hoogsteen (1959, Acta Crystallography 12: 822) first  
15 described the alternate hydrogen bonds present in T-AT  
and C-GC base triads. More recently, G-TA base triads,  
wherein guanine can hydrogen bond with a central  
thymine, have been observed (Griffin et al., 1989,  
Science 245: 967-971). If an oligonucleotide could bind  
20 to a target with both Watson-Crick and alternate  
hydrogen bonds an extremely stable complex would form  
that would have a variety of in vivo and in vitro  
utilities. However, to date there has been no  
disclosure of an oligonucleotide with the necessary  
25 structural features to achieve stable target binding  
with both Watson-Crick and alternate hydrogen bonds.

Oligonucleotides have been observed to bind by  
non-Watson-Crick hydrogen bonding in vitro. For  
example, Cooney et al., 1988, Science 241: 456 disclose  
30 a 27-base single-stranded oligonucleotide which bound to  
a double-stranded nucleic acid via non-Watson-Crick  
hydrogen bonds. However, triple-stranded complexes of

1 this type are not very stable, because the  
oligonucleotide is bound to its target only with less  
stable alternate hydrogen bonds, i.e., without any  
Watson-Crick bonds.

5           Oligonucleotides have been used for a variety  
of utilities. For example, oligonucleotides can be used  
as probes for target nucleic acids that are immobilized  
onto a filter or membrane, or are present in tissues.  
Sambrook et al. (1989, Molecular Cloning: A Laboratory  
10 Manual, Vols. 1-3, Cold Spring Harbor Press, NY) provide  
a detailed review of hybridization techniques.

Furthermore, there has been great interest  
recently in developing oligonucleotides as regulators of  
cellular nucleic acid biological function. This  
15 interest arises from observations on naturally occurring  
complementary, or antisense, RNA used by some cells to  
control protein expression. However, the development of  
oligonucleotides for in vivo regulation of biological  
processes has been hampered by several long-standing  
20 problems, including the low binding stability and  
nuclease sensitivity of linear oligonucleotides.

For example, transcription of the human c-myc  
gene has been inhibited in a cell free, in vitro assay  
system by a 27-base linear oligonucleotide designed to  
25 bind to the c-myc promoter. Inhibition was only  
observed using a carefully controlled in vitro assay  
system wherein lower than physiological temperatures  
were employed, and many cellular enzymes had been  
removed or inactivated. These conditions were necessary  
30 because linear oligonucleotides bind with low affinity  
and are highly susceptible to enzymes which degrade  
linear pieces of DNA (Cooney et al.). Splicing of a

1 pre-mRNA transcript essential for Herpes Simplex virus  
replication has also been inhibited with a linear  
oligonucleotide which was complementary to an acceptor  
splice junction. In this instance, a methylphosphonate  
5 linkage was employed in the linear oligonucleotide to  
increase its nuclease resistance. Addition of this  
chemically-modified oligonucleotide to the growth medium  
caused reduction in protein synthesis and growth of  
uninfected cells, most likely because of toxicity  
10 problems at high concentrations (Smith et al., 1986,  
Proc. Natl. Acad. Sci. USA 83: 2787-2791).

----- In another example, linear oligonucleotides  
were used to inhibit human immunodeficiency virus  
replication in cultured cells. Linear oligonucleotides  
15 complementary to sites within or near the terminal  
repeats of the retrovirus genome and within sites  
complementary to certain splice junctions were most  
effective in blocking viral replication. However, these  
experiments required large amounts of the linear  
20 oligonucleotides before an effect was obtained,  
presumably because of the low binding stability and  
vulnerability of these linear oligonucleotides to  
nucleases (Goodchild et al., 1988, Proc. Natl. Acad.  
Sci. USA 85: 5507-5511).

----- Accordingly, oligonucleotides that are useful  
as regulators of biological processes preferably possess  
certain properties. First, the oligonucleotide should  
bind strongly enough to its complementary target nucleic  
acid to have the desired regulatory effect. Second, it  
30 is generally desirable that the oligonucleotide and its  
target be sequence specific. Third, the oligonucleotide  
should have a sufficient half-life under in vivo

1 conditions for it to be able to accomplish its desired  
regulatory action in the cell. Hence, the  
oligonucleotide should be resistant to enzymes that  
degrade nucleic acids, e.g. nucleases. Fourth, the  
5 oligonucleotide should be able to bind to single- and  
double-stranded targets.

While linear oligonucleotides may satisfy the  
requirement for sequence specificity, linear  
oligonucleotides are sensitive to nucleases and  
10 generally require chemical modification to increase  
biological half-life. Such modifications increase the  
cost of making an oligonucleotide and may present  
toxicity problems. Furthermore, linear oligonucleotides  
bind to form a two-stranded complex like those present  
15 in cellular nucleic acids. Consequently, cellular  
enzymes can readily manipulate and dissociate a linear  
oligonucleotide bound in a double-stranded complex with  
target. The low binding strength and nuclease  
sensitivity of linear oligonucleotides can thus  
20 necessitate administration of high concentrations of  
oligonucleotide, in turn making such administration  
toxic or costly. Moreover, while linear  
oligonucleotides can bind to a double-stranded target  
via alternate hydrogen bonds (e.g. Hoogsteen binding),  
25 linear oligonucleotides cannot readily dissociate a  
~~double-stranded target to replace one strand and thereby~~  
form a more stable Watson-Crick bonding pattern.

Furthermore, increased binding strength  
increases the effectiveness of a regulatory  
30 oligonucleotide. Therefore, an oligonucleotide with  
high binding affinity can be used at lower dosages.  
Lower dosages decrease costs and reduce the likelihood

1           Accordingly, the present invention provides  
single-stranded circular oligonucleotides which, by  
nature of the circularity of the oligonucleotide and the  
domains present on the oligonucleotide, are nuclease  
5 resistant and bind with strong affinity and high  
selectivity to their targeted nucleic acids. Moreover,  
the present circular oligonucleotides can dissociate and  
bind to a double-stranded target without prior  
denaturation of that target.

10           Some types of single-stranded circles of DNA  
or RNA are known. For example, the structures of some  
naturally occurring viral and bacteriophage genomes are  
single-stranded circular nucleic acids. Single-stranded  
circles of DNA have been studied by Erie et al. (1987,  
15 Biochemistry 26: 7150-7159 and 1989, Biochemistry 28:  
268-273). However, none of these circular molecules are  
designed to bind a target nucleic acid. Hence, the  
present invention represents an innovation characterized  
by a substantial improvement relative to the prior art  
20 since the subject circular oligonucleotides exhibit high  
specificity, low or no toxicity and more resistance to  
nucleases than linear oligonucleotides, while binding to  
single- or double-stranded target nucleic acids more  
strongly than conventional linear oligonucleotides.

25

#### SUMMARY OF THE INVENTION:

          The present invention provides a single-  
stranded circular oligonucleotide having at least one  
parallel binding (P) domain and at least one anti-  
30 parallel binding (AP) domain, and having a loop domain  
between each binding domain to form the circular  
oligonucleotide. Each P and corresponding AP domain has

35

1 sufficient complementarity to bind detectably to one  
strand of a defined nucleic acid target with the P  
domain binding in a parallel manner to the target, and  
the AP domain binding in an anti-parallel manner to the  
5 target. Sufficient complementarity means that a  
sufficient number of base pairs exists between the  
target nucleic acid and the P and/or AP domains of the  
circular oligonucleotide to achieve stable, i.e.  
detectable, binding.

10 Another aspect of the present invention  
provides the subject single-stranded circular  
oligonucleotides derivatized with a reporter molecule to  
provide a probe for a target nucleic acid, or with a  
drug or other pharmaceutical agent to provide cell  
15 specific drug delivery, or with agents which can cleave  
or otherwise modify the target nucleic acid or,  
furthermore, with agents that can facilitate cellular  
uptake or target binding of the oligonucleotide.

An additional aspect of the present invention  
20 provides single-stranded circular oligonucleotides  
linked to a solid support for isolation of a nucleic  
acid complementary to the oligonucleotide.

Another aspect of the present invention  
provides a compartmentalized kit for detection or  
25 diagnosis of a target nucleic acid including at least  
one first container providing any one of the present  
circular oligonucleotides. .

A further aspect of the present invention  
provides a method of detecting a target nucleic acid  
30 which involves contacting a single-stranded circular  
oligonucleotide with a sample containing the target  
nucleic acid, for a time and under conditions sufficient

1 to form an oligonucleotide-target complex, and detecting  
the complex. This detection method can be by  
fluorescent energy transfer.

A still further aspect of the present  
5 invention provides a method of regulating biosynthesis  
of a DNA, an RNA or a protein. This method includes  
contacting at least one of the subject circular  
oligonucleotides with a nucleic acid template for the  
DNA, the RNA or the protein under conditions sufficient  
10 to permit binding of the oligonucleotide to a target  
sequence contained in the template, followed by binding  
of the oligonucleotide to the target, blocking access to  
the template and thereby regulating biosynthesis of the  
DNA, the RNA or the protein.

15 An additional aspect of the present invention  
provides pharmaceutical compositions for regulating  
biosynthesis of a nucleic acid or protein containing a  
biosynthesis regulating amount of at least one of the  
subject circular oligonucleotides and a pharmaceutically  
20 acceptable carrier.

A further aspect of the present invention  
provides a method of preparing a single-stranded  
circular oligonucleotide which includes binding a linear  
precircle to an end-joining-oligonucleotide, joining the  
25 two ends of the precircle and recovering the circular  
oligonucleotide product.

Another aspect of the present invention  
provides a method of strand displacement in a double-  
stranded nucleic acid target by contacting the target  
30 with any one of the present circular oligonucleotides  
for a time and under conditions effective to denature  
the target and to bind the circular oligonucleotide.

# 1 BRIEF DESCRIPTIONS OF THE DRAWINGS:

Fig. 1A depicts the bonding patterns of Watson-Crick (anti-parallel domain) AT and GC base pairs. Fig. 1B depicts T-AT, C+GC and G-TA base triads 5 that can form between P, target and AP nucleotides.

Fig. 2 schematically illustrates a circularization reaction for synthesis of single-stranded circular oligonucleotides. A linear precircle oligonucleotide is bound to an oligonucleotide having 10 the same sequence as the target, i.e. an end-joining-oligonucleotide, to form a precircle complex. After ligation, the circularized oligonucleotides are separated from the end-joining-oligonucleotide.

Fig. 3 depicts the sequence of linear 15 precursors to circular oligonucleotides, i.e. precircles (1-3 having SEQ ID NO: 5, SEQ ID NO: 6 and SEQ ID NO: 7), targets (4,5 having SEQ ID NO: 8 and SEQ ID NO: 9), circular oligonucleotides (6,7,8 and 13 having SEQ ID NO: 5-7 and 14), and linear oligonucleotides (9-12 and 20 14 having SEQ ID NO: 10-13 and 15) described in the Examples.

Fig. 4 depicts the structure of a linear precircle complexed with an end-joining-oligonucleotide before ligation.

25 Fig. 5 depicts the effect of pH on circular oligonucleotide:target complex formation as measured by  $T_m$ . Filled circles represent the stability at different pH values for a 6:4 complex while filled squares depict the stability of a 7:5 complex. The sequences of 30 circular oligonucleotides 6 and 7 and targets 4 and 5 are presented in Fig. 3.



1            Fig. 6A depicts the effect of loop size on  
complex formation, with a comparison between binding to  
two targets: a simple (dA)<sub>12</sub> target (squares) and a 36  
nucleotide oligonucleotide target (circles). Fig. 6B  
5 depicts the effect of target and binding domain length  
on complex formation.

Fig. 7 depicts a complex formed between a  
circular oligonucleotide and a target where the P and AP  
binding domains are staggered on the target.

10           Fig. 8 depicts replacement of one strand of a  
fluorescently labeled double stranded target (SEQ ID NO:  
11) by either a linear oligonucleotide having SEQ ID NO:  
8 (dotted line) or a circular oligonucleotide having SEQ  
ID NO: 5 (solid line). Strand replacement was measured  
15 by an increase in fluorescein fluorescence intensity (Y-  
axis) as a function of time (X-axis).

Fig. 9 depicts a plot of observed pseudo-first  
order rate constant,  $K_{obs}$  for duplex target (SEQ ID NO:  
5) at several concentrations. Uncertainty in rate  
20 constants are no more than  $\pm 10\%$ . The depicted curve is  
a rectangular hyperbola generated as a best fit. A  
double reciprocal plot of the data, i.e., [circular  
oligonucleotide]<sup>-1</sup> vs ( $K_{obs}$ )<sup>-1</sup> is linear with a slope of  
 $8.95 \times 10^{-6} \text{ sec} \cdot \text{M}^{-1}$  and a y-intercept of 39.8 sec.

25           DETAILED DESCRIPTION OF THE INVENTION:

The present invention relates to single-  
stranded circular oligonucleotides, i.e. circles, which  
can bind to nucleic acid targets with higher affinity  
30 and selectivity than a corresponding linear  
oligonucleotide. Moreover, since the present circles  
can open up two strands of a double-stranded nucleic

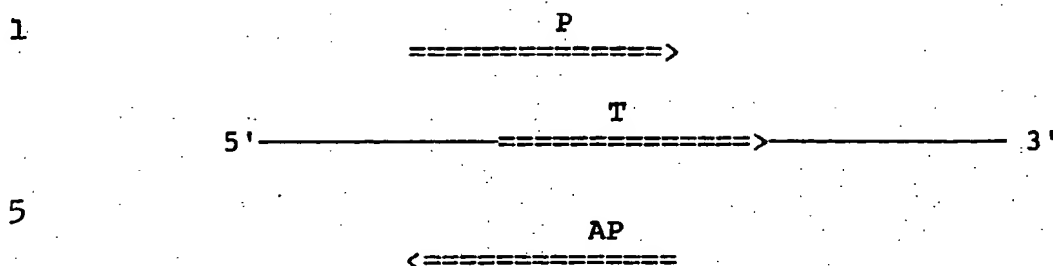
1 acid and bind thereto, both single- and double-stranded  
nucleic acids can be targets for binding by the present  
circular oligonucleotides.

Furthermore, the strong, selective binding of  
5 these circles to either single- or double-stranded  
targets provides a variety of uses, including methods of  
regulating such biological processes as DNA replication,  
RNA transcription, RNA splicing and processing, protein  
translation and the like. Similarly, the ability of  
10 these circles to dissociate double-stranded nucleic  
acids and to selectively and stably bind to targeted  
nucleic acids makes them ideal as diagnostic probes or  
as markers to localize, for example, specific sites in a  
chromosome or other DNA or RNA molecules. Additionally,  
15 the present circles are useful for isolation of  
complementary nucleic acids or for sequence-specific  
delivery of drugs or other molecules into cells.

In particular, the single-stranded circular  
oligonucleotides of the present invention have at least  
20 one parallel binding (P) domain and at least one anti-  
parallel binding (AP) domain and have a loop domain  
between each binding domain, so that a circular  
oligonucleotide is formed. Moreover, each P and AP  
domain exhibits sufficient complementarity to bind to  
25 one strand of a defined nucleic acid target with the P  
domain binding to the target in a parallel manner and  
the AP domain binding to the target in an anti-parallel  
manner.

The schematic illustration set forth below  
30 shows the circular arrangement of one set of P and AP  
oligonucleotide domains relative to each other as well  
as when bound to a target (T, as indicated below).

-12-



The arrows indicate the 5' to 3' orientation of each strand with the 5' end of each domain at the tail and the 3' end at the arrowhead. Hence as used herein

10 binding of nucleic acids in a parallel manner means that the 5'-to-3' orientation is the same for each strand or nucleotide in the complex. This is the type of binding present between the target and the P domain. As used

15 herein, binding of nucleic acids in an anti-parallel manner means that the 5' to 3' orientations of two strands or nucleotides in a complex lie in opposite directions, i.e. the strands are aligned as found in the typical Watson-Crick base pairing arrangement of double helical DNA.

20 When more than one P or AP binding domain is present, such binding domains are separated from other P and AP domains by loop domains whose lengths are sufficient to permit binding to multiple targets.

Moreover, when a circle has multiple AP and P domains,

25 the corresponding targets need not be linked on one nucleic acid strand. Furthermore, a loop domain of a circular oligonucleotide bound to a given target can be an AP or P domain for binding to a second target when the circular oligonucleotide releases from the first

30 target.

35

1           In accordance with this invention, the  
nucleotide sequences of the P and AP domains can be  
det rmined from the defined sequence of the nucleic acid  
target by reference to the base pairing rules provided  
5 hereinbelow. A target can be either single- or double-  
stranded and is selected by its known functional and  
structural characteristics. For example, some preferred  
targets can be coding regions, origins of replication,  
reverse transcriptase binding sites, transcription  
10 regulatory elements, RNA splicing junctions, or ribosome  
binding sites, among others. A target can also be  
selected by its capability for detection or isolation of  
~~a DNA or RNA template.~~ Preferred targets are rich in  
purines, i.e. in adenines and guanines.

15           The nucleotide sequence of the target DNA or  
RNA can be known in full or in part. When the target  
nucleotide sequence is completely known the sequences of  
the P and AP domains are designed with the necessary  
degree of complementarity to achieve binding, as  
20 detected by known procedures, for example by a change in  
light absorption or fluorescence. In some instances,  
the target sequence can be represented by a consensus  
sequence or be only partially known. For example,  
circular oligonucleotides (circles) which bind to an  
25 entire class of targets represented by a consensus  
~~sequence can be provided by designing the P and AP~~  
domains from the target consensus sequence. In this  
instance some of the targets may match the consensus  
sequence exactly and others may have a few mismatched  
30 bases, but not enough mismatch to prevent binding.  
Likewise, if a portion of a target sequence is known,  
one skilled in the art can refer to the base pairing

1 rules provided hereinbelow to design circles which bind  
to that target with higher affinity than a linear  
oligonucleotide that has a sequence corresponding to  
that of the circle.

5           Thus, the present invention is also directed  
to circles having P and AP domains which are  
sufficiently complementary to bind to a nucleic acid  
target wherein a sufficient number, but not necessarily  
all, nucleotide positions in the P and AP domains are  
10 determined from the target sequence in accordance with  
the base pairing rules of this invention. The number of  
determined (i.e. known) positions is that number of  
positions which are necessary to provide sufficient  
complementarity for binding of the subject  
15 oligonucleotides to their targets, as detected by  
standard procedures including a change in light  
absorption upon binding or melting.

The base pairing rules of the present  
invention provide for the P domain to bind to the target  
20 by forming base pairs wherein the P domain and target  
nucleotides have the same 5' to 3' orientation. In  
particular, these rules are satisfied to the extent  
needed to achieve binding of a circular oligonucleotide  
to its nucleic acid target, i.e. the degree of

25 complementarity need not be 100% so long as binding can  
be detected. Hence, the general rules for determining  
the sequence of the P domain are thus:

when a base for a position in the target is  
guanine or a guanine analog, then P has cytosine, or a  
30 suitable analog thereof, in a corresponding position;

when a base for a position in the target is  
adenine, or an adenine analog then P has thymine or

1 uracil, or suitable analogs thereof, in a corresponding position;

when a base for a position in the target is thymine, or a thymine analog, then P has cytosine or 5 guanine, or suitable analogs thereof, in a corresponding position;

when a base for a position in the target is cytosine, or a cytosine analog, then P has cytosine, thymine or uracil, or suitable analogs thereof, in a 10 corresponding position; and

when a base for a position in the target is uracil, or a uracil analog, then P has cytosine, guanine, thymine, or uracil, or suitable analogs thereof, in a corresponding position.

15 The base pairing rules of the present invention provide for the AP domain to bind to the target by forming base pairs wherein the AP domain and target nucleotides are oriented in opposite directions. In particular these rules are satisfied to the extent 20 necessary to achieve detectable binding of a circular oligonucleotide to its nucleic acid target, i.e. the degree of complementarity can be less than 100%. Hence, the base pairing rules can be adhered to only insofar as is necessary to achieve sufficient complementarity for 25 binding to be detected between the circular oligonucleotide and its target.

Thus, the general rules for determining the sequence of the AP domain are as follows:

when a base for a position in the target is 30 guanine, or a guanine analog, then AP has cytosine or uracil, or suitable analogs thereof, in a corresponding position;

1 when a base for a position in the target is adenine, or an adenine analog, then AP has thymine or uracil, or suitable analogs thereof, in a corresponding position;

5 when a base for a position in the target is thymine, or a thymine analog, then AP has adenine, or a suitable analog thereof, in a corresponding position; and

when a base for a position in the target is  
10 cytosine, or a cytosine analog, then AP has a guanine, or a suitable analog thereof, in corresponding position;

when a base for a position in the target is  
uracil, or a uracil analog, then AP has adenine or  
guanine, or suitable analogs thereof, in a corresponding  
15 position.

In a preferred embodiment, the P, AP and loop domains are not complementary to each other.

Table 1 summarizes which nucleotides can form anti-parallel base pairs or parallel base pairs with a  
20 defined target nucleotide.

Table 1

| Target Nucleotide <sup>a</sup> | Anti-Parallel Domain Nucleotide <sup>a</sup> | Parallel Domain Nucleotide <sup>a</sup> |
|--------------------------------|--|---|
| G                              | C or U                                       | C                                       |
| A                              | T or U                                       | T or U                                  |
| T                              | A  | C or G                                  |
| C                              | G  | C, T or U                               |
| U                              | A or G                                       | C, G, T or U                            |

30 <sup>a</sup> Or a suitable analog

35

- 1 Two complementary single-stranded nucleic acids form a stable double helix (duplex) when the strands bind, or hybridize, to each other in the typical Watson-Crick fashion, i.e. via anti-parallel GC and AT  
5 base pairs. For the present invention, stable duplex formation and stable triplex formation is achieved when the P and AP domains exhibit sufficient complementarity to the target sequence to achieve stable binding between the circular oligonucleotide and the target molecule.
- 10 Stable binding occurs when an oligonucleotide remains detectably bound to target under the required conditions.

---

Complementarity between nucleic acids is the degree to which the bases in one nucleic acid strand can  
15 hydrogen bond, or base pair, with the bases in a second nucleic acid strand. Hence, complementarity can sometimes be conveniently described by the percentage, i.e. proportion, of nucleotides which form base pairs between two strands or within a specific region or  
20 domain of two strands. For the present invention sufficient complementarity means that a sufficient number of base pairs exist between a target nucleic acid and the P and/or AP domains of the circular oligonucleotide to achieve detectable binding.

---

25 Moreover, the degree of complementarity between the P domain and the target and the AP domain and the target need not be the same. When expressed or measured by percentage of base pairs formed, the degree of complementarity can range from as little as about 30-40%  
30 complementarity to full, i.e. 100%, complementarity. In general, the overall degree of complementarity between the P or AP domain and the target is preferably at least



1 about 50%. However, the P domain can sometimes have  
less complementarity with the target than the AP domain  
has with the target, for example the P domain can have  
about 30% complementarity with the target while the AP  
5 domain can have substantially more complementarity, e.g.  
50% to 100% complementarity.

Moreover, the degree of complementarity that  
provides detectable binding between the subject circular  
oligonucleotides and their respective targets, is  
10 dependent upon the conditions under which that binding  
occurs. It is well known that binding, i.e.

hybridization, between nucleic acid strands depends on  
factors besides the degree of mismatch between two  
sequences. Such factors include the GC content of the  
15 region, temperature, ionic strength, the presence of  
formamide and types of counter ions present. The effect  
that these conditions have upon binding is known to one  
skilled in the art. Furthermore, conditions are  
frequently determined by the circumstances of use. For  
20 example, when a circular oligonucleotide is made for use  
in vivo, no formamide will be present and the ionic  
strength, types of counter ions, and temperature  
correspond to physiological conditions. Binding  
conditions can be manipulated in vitro to optimize the

25 utility of the present oligonucleotides. A thorough  
treatment of the qualitative and quantitative  
considerations involved in establishing binding  
conditions that allow one skilled in the art to design  
appropriate oligonucleotides for use under the desired  
30 conditions is provided by Beltz et al., 1983, Methods  
Enzymol. 100: 266-285 and by Sambrook et al.

1           Thus for the pr sent invention, one of  
ordinary skill in the art can readily design a  
nucleotide sequence for the P and AP domains of the  
subject circular oligonucleotides which exhibits  
5 sufficient complementarity to detectably bind to its  
target sequence. As used herein "binding" or "stable  
binding" means that a sufficient amount of the  
oligonucleotide is bound or hybridized to its target to  
permit detection of that binding. Binding can be  
10 detected by either physical or functional properties of  
the target:circular oligonucleotide complex.

Binding between a target and an  
oligonucleotide can be detected by any procedure known  
to one skilled in the art, including both functional or  
15 physical binding assays. Binding may be detected  
functionally by determining whether binding has an  
observable effect upon a biosynthetic process such as  
DNA replication, RNA transcription, protein translation  
and the like.

20           Physical methods of detecting the binding of  
complementary strands of DNA or RNA are well known in  
the art, and include such methods as DNase I or chemical  
footprinting, gel shift and affinity cleavage assays and  
light absorption detection procedures. For example, a  
25 method which is widely used, because it is so simple and  
reliable, involves observing a change in light  
absorption of a solution containing an oligonucleotide  
and a target nucleic acid at 220 to 300 nm as the  
temperature is slowly increased. If the oligonucleotide  
30 has bound to its target, there is a sudden increase in  
absorption at a characteristic temperature as the  
oligonucleotide and target dissociate or melt.

1           The binding between an oligonucleotide and its  
target nucleic acid is frequently characterized by the  
temperature at which 50% of the oligonucleotide is  
melted from its target. This temperature is the melting  
5 temperature ( $T_m$ ). A higher  $T_m$  means a stronger or more  
stable complex relative to a complex with a lower  $T_m$ .  
The stability of a duplex increases with increasing G:C  
content since G:C base pairs have three hydrogen bonds  
whereas A:T base pairs have two. The circular  
10 oligonucleotides of the present invention provide  
additional hydrogen bonds and hence more stability since  
two binding domains are available for bonding to a  
single target nucleic acid, i.e. the P domain and the AP  
domain. Hence, the triplex formed by a circular  
15 oligonucleotide bound to its target nucleic acid should  
melt at a higher  $T_m$  than the duplex formed by a linear  
oligonucleotide and a target.

Circular oligonucleotides bind to a nucleic  
acid target through hydrogen bonds formed between the  
20 nucleotides of the binding domains and the target. The  
AP domain can bind by forming Watson-Crick hydrogen  
bonds (Fig. 1). The P domain can bind to the target  
nucleotides by forming non-Watson-Crick hydrogen bonds  
(e.g., Fig. 1 and Table I). When two nucleotides from  
25 different strands of DNA or RNA hydrogen bond by the  
base pairing rules defined herein, a base pair or duplex  
is formed. When a nucleotide from AP and a nucleotide  
from P both bind to the same target nucleotide, a base  
triad is formed.

30           Parallel domain base pairing with a  
complementary target strand of nucleic acid, is  
thermodynamically less favorable than Watson-Crick base

1 pairing; however, when both parallel and antiparallel  
pairing modes are present in a single molecule, highly  
stable complex s can form. Thus, two opposing domains  
of a circular oligomer form a complex with a central  
5 target, giving a triplex structure, or a triple helical  
complex, bounded by the two looped ends of the circle.  
For example, this arrangement can allow formation of up  
to four hydrogen bonds when two thymines bind to a  
target adenine and up to five hydrogen bonds when two  
10 cytosines bind to a target guanine.

Furthermore, because of the binding  
characteristics of the P and AP domains, the present  
circular oligonucleotides have a higher selectivity for  
a particular target than do corresponding linear  
15 oligonucleotides. At least two factors can contribute  
to this high selectivity. First, circular  
oligonucleotides of this invention bind twice to the  
same central target strand. Hence two domains are  
involved in selecting a target. Second, protonation of  
20 cytosine in a C+G-C triad is favored only when this  
triad forms and the additional proton gives the triad a  
positive charge. This positive charge can lessen the  
negative charge repulsions arising from the  
juxtapositioning of three phosphodiester backbones.

25 Unlike linear oligonucleotides, the present  
circular oligonucleotides can displace one strand of a  
double-stranded target under conditions where  
denaturation of the double-stranded target is  
thermodynamically unfavorable. Linear oligonucleotides  
30 do not have this capacity to displace a strand of a  
duplex. For example, the half-life of a double-stranded  
target in the presence of a complementary linear

1 oligonucleotide is about 58 min i. . so long that the  
linear oligonucleotide has little utility for displacing  
one strand of the duplex target. However, a double-  
stranded target has a half-life of only 30 sec in the  
5 presence of the present circular oligonucleotides.  
Therefore, the circular oligonucleotides of the present  
invention have utility not only for binding single-  
stranded targets, but also for binding to double-  
stranded targets. Accordingly, since both single- and  
10 double-stranded nucleic acids are available as targets  
for the present circular oligonucleotides, these  
circular oligonucleotides can have greater utility than  
linear oligonucleotides. For example, the present  
circular oligonucleotides are better regulators of  
15 biological processes in vivo and better in vitro  
diagnostic probes than corresponding linear  
oligonucleotides.

When the nucleic acid template extends beyond  
the central triple-stranded target:circle complex, a P  
20 or an AP domain may bind as duplex on either side of the  
triple standard complex. Hence a target:circular  
oligonucleotide complex can be partially two stranded  
and partially three-stranded, wherein two-stranded  
portions can be P:target duplexes, without bound AP  
25 nucleotides, or AP:target duplexes, without bound P  
nucleotides. This binding arrangement is a staggered  
binding arrangement.

Each P domain, AP domain and target can  
independently have about 2 to about 200 nucleotides with  
30 preferred lengths being about 4 to about 100  
nucleotides. The most preferred lengths are 6 to 36  
nucleotides.

1           The P and AP domains are separated by loop  
domains which can independently have from about 2 to  
about 2000 nucleotides. A preferred loop length is from  
about 3 to about 8 nucleotides with an especially  
5 preferred length being about 5 nucleotides.

          According to the present invention, the loop  
domains do not have to be composed of nucleotide bases.  
Non-nucleotide loops can make the present circular  
oligonucleotides cheaper to produce. More  
10 significantly, circular oligonucleotides with non-  
nucleotide loops are more resistant to nucleases and  
therefore have a longer biological half-life than  
linear oligonucleotides. Furthermore, loops having no  
charge, or a positive charge, can be used to promote  
15 binding by eliminating negative charge repulsions  
between the loop and target. In addition, circular  
oligonucleotides having uncharged or hydrophobic non-  
nucleotide loops can penetrate cellular membranes better  
than circular oligonucleotides with nucleotide loops.

20           As contemplated herein, non-nucleotide loop  
domains can be composed of alkyl chains, polyethylene  
glycol or oligoethylene glycol chains or other chains  
providing the necessary steric or flexibility properties  
which are compatible with oligonucleotide synthesis.  
25 The length of these chains is equivalent to about 2 to  
about 2000 nucleotides, with preferred lengths  
equivalent to about 3 to about 8 nucleotides. The most  
preferred length for these chains is equivalent to about  
5 nucleotides.

30           Preferred chains for non-nucleotide loop  
domains are polyethylene glycol or oligoethylene glycol  
chains. In particular, oligoethylene glycol chains

1 having a length similar to a 5 nucleotide chain, e.g. a pentaethylene glycol, a hexaethylene glycol or a heptaethylene glycol chain, are preferred.

The circular oligonucleotides are single-  
5 stranded DNA or RNA, with the bases guanine (G), adenine (A), thymine (T), cytosine (C) or uracil (U) in the nucleotides, or with any nucleotide analog that is capable of hydrogen bonding in a parallel or anti-parallel manner. Nucleotide analogs include  
10 pseudocytidine, isopseudocytidine, 3-aminophenyl-imidazole, 2'-O-methyl-adenosine, 7-deazadenosine, 7-deazaguanosine, 4-acetylcytidine, 5-(carboxy-hydroxymethyl)-uridine, 2'-O-methylcytidine, 5-carboxymethylaminomethyl-2-thioridine, 5-  
15 carboxymethylamino-methyluridine, dihydrouridine, 2'-O-methyluridine, 2'-O-methyl-pseudouridine, beta,D-galactosylqueosine, 2'-O-methylguanosine, inosine, N6-isopentenyladenosine, 1-methyladenosine, 1-methyl-pseudouridine, 1-methylguanosine, 1-methylinosine, 2,2-  
20 dimethylguanosine, 2-methyladenosine, 2-methylguanosine, 3-methylcytidine, 5-methylcytidine, 5-methyluridine, N6-methyl-adenosine, 7-methylguanosine, 5-methylamino-methyluridine, 5-methoxyaminomethyl-2-thiouridine, 8-D-mannosylqueosine, 5-methoxycarbonylmethyluridine, 5-  
25 methoxyuridine, 2-methyl-thio-N6-isopentenyladenosine, N-(9-beta-D-ribofuranosyl-2-methylthiopurine-6-yl)-carbamoyl)threonine, N-(9-beta-D-ribofuranosylpurine-6-yl)-N-methylcarbamoyl)threonine. When possible, either ribose or deoxyribose sugars can be used with these  
30 analogs. Nucleotides bases in an  $\alpha$ -anomeric conformation can also be used in the circular oligonucleotides of the present invention.

1 Preferred nucleotide analogs are unmodified G,  
A, T, C and U nucleotides; pyrimidine analogs with lower  
alkyl, lower alkoxy, lower alkylamine, phenyl or lower  
alkyl substituted phenyl groups in the 5 position of the  
5 base and purine analogs with similar groups in the 7 or  
8 position of the base. Especially preferred nucleotide  
analogs are 5-methylcytosine, 5-methyluracil,  
diaminopurine, and nucleotides with a 2'-O-methylribose  
moiety in place of ribose or deoxyribose. As used  
10 herein lower alkyl, lower alkoxy and lower alkylamine  
contain from 1 to 6 carbon atoms and can be straight  
chain or branched. These groups include methyl, ethyl,  
propyl, isopropyl, butyl, isobutyl, tertiary butyl,  
amyl, hexyl and the like. A preferred alkyl group is  
15 methyl.

Circular oligonucleotides can be made first as  
linear oligonucleotides and then circularized. Linear  
oligonucleotides can be made by any of a myriad of  
procedures known for making DNA or RNA oligonucleotides.  
20 For example, such procedures include enzymatic synthesis  
and chemical synthesis.

Enzymatic methods of DNA oligonucleotide  
synthesis frequently employ Klenow, T7, T4, Taq or E.  
coli DNA polymerases as described in Sambrook et al.  
25 Enzymatic methods of RNA oligonucleotide synthesis  
frequently employ SP6, T3 or T7 RNA polymerase as  
described in Sambrook et al. Reverse transcriptase can  
also be used to synthesize DNA from RNA (Sambrook  
et al.). To prepare oligonucleotides enzymatically  
30 requires a template nucleic acid which can either be  
synthesized chemically, or be obtained as mRNA, genomic  
DNA, cloned genomic DNA, cloned cDNA or other



1 recombinant DNA. Some enzymatic methods of DNA  
oligonucleotide synthesis can require an additional  
primer oligonucleotide which can be synthesized  
chemically. Finally, linear oligonucleotides can be  
5 prepared by PCR techniques as described, for example, by  
Saiki et al., 1988, Science 239:487.

Chemical synthesis of linear oligonucleotides  
is well known in the art and can be achieved by solution  
or solid phase techniques. Moreover, linear  
10 oligonucleotides of defined sequence can be purchased  
commercially or can be made by any of several different  
synthetic procedures including the phosphoramidite,  
phosphite triester, H-phosphonate and phosphotriester  
methods, typically by automated synthesis methods. The  
15 synthesis method selected can depend on the length of  
the desired oligonucleotide and such choice is within  
the skill of the ordinary artisan. For example, the  
phosphoramidite and phosphite triester method produce  
oligonucleotides having 175 or more nucleotides while  
20 the H-phosphonate method works well for oligonucleotides  
of less than 100 nucleotides. If modified bases are  
incorporated into the oligonucleotide, and particularly  
if modified phosphodiester linkages are used, then the  
synthetic procedures are altered as needed according to  
25 known procedures. In this regard, Uhlmann et al. (1990,  
Chemical Reviews 90: 543-584) provide references and  
outline procedures for making oligonucleotides with  
modified bases and modified phosphodiester linkages.

Synthetic, linear oligonucleotides may be  
30 purified by polyacrylamide gel electrophoresis, or by  
any of a number of chromatographic methods, including  
gel chromatography and high pressure liquid

1 chromatography. To confirm a nucleotide sequence,  
oligonucleotides may be subjected to DNA sequencing by  
any of the known procedures, including Maxam and Gilbert  
sequencing, Sanger sequencing, capillary electrophoresis  
5 sequencing the wandering spot sequencing procedure or by  
using selective chemical degradation of oligonucleotides  
bound to Hybond paper. Sequences of short  
oligonucleotides can also be analyzed by plasma  
desorption mass spectroscopy or by fast atom bombardment  
10 (McNeal, et al., 1982, J. Am. Chem. Soc. 104: 976;  
Viari, et al., 1987, Biomed. Environ. Mass Spectrom. 14:  
83; Grotjahn et al., 1982, Nuc. Acid Res. 10: 4671).  
Sequencing methods are also available for RNA  
oligonucleotides.

15 The present invention provides several methods  
of preparing circular oligonucleotides from linear  
precursors (i.e. precircles), including a method wherein  
a precircle is synthesized and bound to an end-joining-  
oligonucleotide and the two ends of the precircle are  
20 joined. Any method of joining two ends of an  
oligonucleotide is contemplated by the present  
invention, including chemical methods employing, for  
example, known coupling agents like BrCN, N-  
cyanoimidazole  $ZnCl_2$ , 1-ethyl-3-(3-  
25 dimethylaminopropyl)carbodiimide and other carbodimides  
and carbonyl diimidazoles. Furthermore, the ends of a  
precircle can be joined by condensing a 5' phosphate and  
a 3' hydroxy, or a 5' hydroxy and a 3' phosphate.

In accordance with the present invention, a  
30 simple one-step chemical method is provided to construct  
the subject circular oligonucleotides, or circles, from  
precircles. An oligonucleotide is constructed which has

1 the same sequence as the target nucleic acid; this is  
the end-joining oligonucleotide. A DNA or RNA linear  
precircle is chemically or enzymatically synthesized and  
phosphorylated on its 5' or 3' end, again by either  
5 chemical or enzymatic means. The precircle and the end-  
joining oligonucleotide are mixed and annealed, thereby  
forming a complex in which the 5' and 3' ends of the  
precircle are adjacent, as depicted in Fig. 2. It is  
preferred that the ends of the precircle fall within a  
10 binding domain, not within a loop, and preferably within  
the anti-parallel binding domain rather than the  
parallel domain. Moreover, it is preferred that a  
precircle have a 3'-phosphate rather than a 5'-  
phosphate. After complex formation, the ends undergo a  
15 condensation reaction in a buffered aqueous solution  
containing divalent metal ions and BrCN at about pH 7.0.  
In a preferred embodiment the buffer is imidazole-Cl at  
pH 7.0 with a divalent metal such as Ni, Zn, Mn, or Co.  
Ni is the most preferred divalent metal. Condensation  
20 occurs after about 6-48 hr. of incubation at 4-37°C.  
Other divalent metals, such as Cu, Pb, Ca and Mg, can  
also be used.

One method for RNA circularization  
incorporates the appropriate nucleotide sequences,  
25 preferably in a loop domain, into an RNA oligonucleotide  
to promote self splicing, since a circular product is  
formed under the appropriate conditions (Sugimoto  
et al., 1988, Biochemistry: 27: 6384-6392).

Enzymatic circle closure is also possible  
30 using DNA ligase or RNA ligase under conditions  
appropriate for these enzymes.

1           Circular oligonucleotides can be separated  
from the template by denaturing gel electrophoresis or  
melting followed by gel electrophoresis, size selective  
chromatography, or other appropriate chromatographic or  
5 electrophoretic methods. The recovered circular  
oligonucleotide can be further purified by standard  
techniques as needed for its use in the methods of the  
present invention.

          The present invention also contemplates  
10 derivatization or chemical modification of the subject  
oligonucleotides with chemical groups to facilitate  
cellular uptake. For example, covalent linkage of a  
cholesterol moiety to an oligonucleotide can improve  
cellular uptake by 5- to 10- fold which in turn improves  
15 DNA binding by about 10- fold (Boutorin et al., 1989,  
FEBS Letters 254: 129-132). Other ligands for cellular  
receptors may also have utility for improving cellular  
uptake, including, e.g. insulin, transferrin and others.  
Similarly, derivatization of oligonucleotides with poly-  
20 L-lysine can aid oligonucleotide uptake by cells  
(Schell, 1974, Biochem. Biophys. Acta 340: 323, and  
Lemaitre et al., 1987, Proc. Natl. Acad. Sci. USA 84:  
648). Certain protein carriers can also facilitate  
cellular uptake of oligonucleotides, including, for  
25 example, serum albumin, nuclear proteins possessing  
signals for transport to the nucleus, and viral or  
bacterial proteins capable of cell membrane penetration.  
Therefore, protein carriers are useful when associated  
with or linked to the circular oligonucleotides of this  
30 invention. Accordingly, the present invention  
contemplates derivatization of the subject circular  
oligonucleotides with groups capable of facilitating

1 cellular uptake, including hydrocarbons and non-polar  
groups, cholesterol, poly-L-lysine and proteins, as well  
as other aryl or steroid groups and polycations having  
analogous beneficial effects, such as phenyl or naphthyl  
5 groups, quinoline, anthracene or phenanthracene groups,  
fatty acids, fatty alcohols and sesquiterpenes,  
diterpenes and steroids.

The present invention further contemplates  
derivatization of the subject oligonucleotides with  
10 agents that can cleave or modify the target nucleic acid  
or other nucleic acid strands associated with or in the  
vicinity of the target. For example, viral DNA or RNA  
can be targeted for destruction without harming cellular  
nucleic acids by administering a circular  
15 oligonucleotide complementary to the targeted nucleic  
acid which is linked to an agent that, upon binding, can  
cut or render the viral DNA or RNA inactive. Nucleic  
acid destroying agents that are contemplated by the  
present invention as having cleavage or modifying  
20 activities include, for example, RNA and DNA nucleases,  
ribozymes that can cleave RNA, azidoproflavine,  
acridine, EDTA/Fe, chloroethylamine, azidophenacyl and  
phenanthroline/Cu. Uhlmann et al. (1990, Chemical  
Reviews 90: 543-584) provide further information on the  
25 use of such agents and methods of derivatizing  
oligonucleotides that can be adapted for use with the  
subject circular oligonucleotides.

Derivatization of the subject circular  
oligonucleotides with groups that facilitate cellular  
30 uptake or target binding, as well as derivatization with  
nucleic acid destroying agents or drugs, can be done by  
any of the procedures known to one skilled in the art.

1 Moreover, the desired groups can be added to nucleotid s  
before synthesis of the oligonucleotide. For example,  
these groups can be linked to the 5-position of T or C  
and these modified T and C nucleotides can be used for  
5 synthesis of the present circular oligonucleotides. In  
addition, derivatization of selected nucleotides permits  
incorporation of the group into selected domains of the  
circular oligonucleotide. For example, in some  
instances it is preferable to incorporate certain groups  
10 into a loop where that group will not interfere with  
binding, or into an AP or P domain to facilitate  
cleavage or modification of the target nucleic acid.

In accordance with the present invention,  
modification in the phosphodiester backbone of circular  
15 oligonucleotides is also contemplated. Such  
modifications can aid uptake of the oligonucleotide by  
cells or can extend the biological half-life of such  
nucleotides. For example, circular oligonucleotides may  
penetrate the cell membrane more readily if the negative  
20 charge on the internucleotide phosphate is eliminated.  
This can be done by replacing the negatively charged  
phosphate oxygen with a methyl group, an amine or by  
changing the phosphodiester linkage into a  
phosphotriester linkage by addition of an alkyl group to  
25 the negatively charged phosphate oxygen. Alternatively,  
one or more of the phosphate atoms which is part of the  
normal phosphodiester linkage can be replaced. For  
example, NH-P, CH<sub>2</sub>-P or S-P linkages can be formed.  
Accordingly, the present invention contemplates using  
30 methylphosphonates, phosphorothioates,  
phosphorodithioates, phosphotriesters and phosphorus-  
boron (Sood et al., 1990, J. Am. Chem. Soc. 112: 9000)

1 linkages. The phosphodiester group can be replaced with  
siloxane, carbonate, acetamidate or thioether groups.  
These modifications can also increase the resistance of  
the subject oligonucleotides to nucleases. Methods for  
5 synthesis of oligonucleotides with modified  
phosphodiester linkages are reviewed by Uhlmann et al.

Circular oligonucleotides with non-nucleotide  
loops can be prepared by any known procedure. For  
example, Durand et al. (1990, Nucleic Acids Res. 18:  
10 6353-6359) provides synthetic procedures for linking  
non-nucleotide chains to DNA. Such procedures can  
generally be adapted to permit an automated synthesis of  
a linear oligonucleotide precursor which is then used to  
make a circular oligonucleotide of the present  
15 invention. In general, groups reactive with nucleotides  
in standard DNA synthesis, e.g. phosphoramidite, H-  
phosphonate, dimethoxytrityl, monomethoxytrityl and the  
like, can be placed at the ends of non-nucleotide chains  
and nucleotides corresponding to the ends of P and AP  
20 domains can be linked thereto.

Additionally, different nucleotide sugars can  
be incorporated into the oligonucleotides of this  
invention. For example, RNA oligonucleotides can be  
used since RNA:DNA hybrids are more stable than DNA:DNA  
25 hybrids. Additional binding stability can also be  
provided by using 2'-O-methyl ribose in the present  
circular oligonucleotides. Phosphoramidite chemistry  
can be used to synthesize RNA oligonucleotides as  
described (Reese, C. B. In Nucleic Acids & Molecular  
30 Biology; Springer-Verlag: Berlin, 1989; Vol. 3, p. 164;  
and Rao, et al., 1987, Tetrahedron Lett. 28: 4897).

1           The synthesis of RNA 2'-O-methyl-  
oligoribonucleo-tides and DNA oligonucleotides differ  
only slightly. RNA 2'-O-methyloligonucleotides can be  
prepared with minor modifications of the amidite, H-  
5 phosphonate or phosphotriester methods (Shibahara et al,  
1987, Nucleic Acids Res. 15: 4403; Shibahara et al.,  
1989, Nucleic Acids Res. 17: 239; Anoue et al., 1987,  
Nucleic Acids Res. 15: 6131).

          In another embodiment the present invention,  
10 circular oligonucleotides can accelerate the  
dissociation of a double-stranded nucleic acid target.  
Therefore the double-stranded nucleic acid target does  
not have to be subjected to denaturing conditions before  
binding of the present circular oligonucleotides. Thus,  
15 the circular oligonucleotides can bind to both single-  
and double-stranded nucleic acid targets under a wider  
variety of conditions, and particularly under  
physiological conditions. The present circular  
oligonucleotides are several orders of magnitude faster  
20 at accelerating duplex nucleic acid strand displacement  
than are the corresponding linear oligonucleotides.

          The present invention therefore provides a  
means to displace one strand of a double-stranded  
nucleic acid target with one of the subject circular  
25 oligonucleotides without the necessity of prior  
denaturation of the double-stranded nucleic acid target.  
Thus, the present invention provides a method of strand  
displacement in a double-stranded nucleic acid target by  
contacting the target with one of the subject circular  
30 oligonucleotides for a time and under conditions  
effective to denature said target and permit the  
circular oligonucleotide to bind to the target. The



1 target for the present circular oligonucleotides can be  
a double-stranded nucleic acid, either RNA or DNA, which  
has not undergone denaturation by, for example, heating  
or exposure to alkaline pH.

5 As used herein, the nucleic acids for strand  
displacement can be present in an organism or present in  
a sample which includes an impure or pure nucleic acid  
preparation, a tissue section, a prokaryotic or  
eukaryotic cell smear, a chromosomal squash and the  
10 like. Moreover, the nucleic acid targets for strand  
displacement by the present circular oligonucleotides  
include viral, bacterial, fungal or mammalian nucleic  
acids.

According to the present invention, conditions  
15 effective to denature the target by strand displacement  
and thereby permit binding, include having a suitable  
circular oligonucleotide to target nucleic acid ratio.  
Moreover, as used herein a suitable ratio of circular  
oligonucleotide to target is about 1 to about 100, and  
20 is preferably about 1 to about 50.

Moreover, as used herein a time effective to  
denature a double-stranded nucleic acid by strand-  
displacement with an oligonucleotide of the present  
invention is about 1 minute to about 16 hours.

25 A circular oligonucleotide can associate with  
a duplex target by first binding in the P domain. Such  
P domain binding juxtaposes the AP domain nucleotides to  
compete for Watson-Crick binding to target nucleotide.  
This P domain pre-association followed by AP domain  
30 nucleotide competition for Watson-Crick binding may form  
the basis for the observed acceleration in strand  
displacement by circular oligonucleotides.

1           In summary, the subject circular  
oligonucleotides have three important features which  
enable duplex strand displacement. First, the circular  
oligonucleotide has the ability to preassociate, which  
5 results in a high local concentration. Second, the  
circular oligonucleotide contains a second (AP) binding  
domain, which competes for binding to a complementary  
strand of the duplex. Finally, the circular  
oligonucleotide binds with higher affinity than the  
10 displaced strand of the duplex, thereby driving the  
reaction to completion.

The present invention contemplates a variety  
of utilities for the subject circular oligonucleotides  
which are made possible by their selective and stable  
15 binding properties with both single- and double-stranded  
targets. Some utilities include, but are not limited  
to: use of circular oligonucleotides of defined  
sequence, bound to a solid support, for affinity  
isolation of complementary nucleic acids; use of the  
20 subject oligonucleotides to provide sequence specific  
stop signals during polymerase chain reaction (PCR);  
covalent attachment of a drug, drug analog or other  
therapeutic agent to circular oligonucleotides to allow  
cell type specific drug delivery; labeling circular  
25 oligonucleotides with a detectable reporter molecule for  
~~localizing, quantitating or identifying complementary~~  
target nucleic acids; and binding circular  
oligonucleotides to a cellular or viral nucleic acid  
template and regulating biosynthesis directed by that  
30 template.

The subject circular oligonucleotides can be  
attached to a solid support such as silica, cellulose,

1 nylon, and other natural or synthetic materials that are  
used to make beads, filters, and column chromatography  
resins. Attachment procedures for nucleic acids to  
solid supports of these types are well known; any known  
5 attachment procedure is contemplated by the present  
invention. A circular oligonucleotide attached to a  
solid support can then be used to isolate a  
complementary nucleic acid. Isolation of the  
complementary nucleic acid can be done by incorporating  
10 the oligonucleotide:solid support into a column for  
chromatographic procedures. Other isolation methods can  
be done without incorporation of the  
oligonucleotide:solid support into a column, e.g. by  
utilization of filtration procedures. Circular  
15 oligonucleotide:solid supports can be used, for example,  
to isolate poly(A)<sup>+</sup> mRNA from total cellular or viral  
RNA by making a circular oligonucleotide with P and AP  
domain poly(dT) or poly(U) sequences. Circular  
oligonucleotides are ideally suited to applications of  
20 this type because they are nuclease resistant and bind  
target nucleic acids so strongly.

Further utilities are available for the  
subject oligonucleotides in the field of polymerase  
chain reaction (PCR) technology. PCR technology  
25 provides methods of synthesizing a double-stranded DNA  
fragment encoded in a nucleic acid template between two  
known nucleic acid sequences which are employed as  
primer binding sites. In some instances it is desirable  
to produce a single-stranded DNA fragment before or  
30 after having made some of the double stranded fragment.  
This can be done by, for example, binding a circular  
oligonucleotide of the present invention to one of the

1 primer binding sites or to a site lying between the  
primer binding sites.

The present invention also contemplates using  
the subject circular oligonucleotides for targeting  
5 drugs to specific cell types. Such targeting can allow  
selective destruction or enhancement of particular cell  
types, e.g. inhibition of tumor cell growth can be  
attained. Different cell types express different genes,  
so that the concentration of a particular mRNA can be  
10 greater in one cell type relative to another cell type,  
such an mRNA is a target mRNA for cell type specific  
drug delivery by circular oligonucleotides linked to  
drugs or drug analogs. Cells with high concentrations  
of target mRNA are targeted for drug delivery by  
15 administering to the cell a circular oligonucleotide  
with a covalently linked drug that is complementary to  
the target mRNA.

The present invention also contemplates  
labeling the subject circular oligonucleotides for use  
20 as probes to detect a target nucleic acid. Labelled  
circular oligonucleotide probes have utility in  
diagnostic and analytical hybridization procedures for  
localizing, quantitating or detecting a target nucleic  
acid in tissues, chromosomes or in mixtures of nucleic  
25 acids. Circular oligonucleotide probes of this invention  
~~represent a substantial improvement over linear nucleic~~  
acid probes because the circular oligonucleotides can  
replace one strand of a double-stranded nucleic acid,  
and because the present oligonucleotides have two  
30 binding domains which not only provide increased binding  
stability but also impart a greater sequence selectivity

1 (or specificity) for the target:oligonucleotide  
interaction.

Labeling of a circular oligonucleotide can be  
done by incorporating nucleotides linked to a "reporter  
5 molecule" into the subject circular oligonucleotides. A  
"reporter molecule", as defined herein, is a molecule or  
atom which, by its chemical nature, provides an  
identifiable signal allowing detection of the circular  
oligonucleotide. Detection can be either qualitative or  
10 quantitative. The present invention contemplates using  
any commonly used reporter molecule including  
radionuclides, enzymes, biotins, psoralens,  
fluorophores, chelated heavy metals, and luciferin. The  
most commonly used reporter molecules are either  
15 enzymes, fluorophores or radionuclides linked to the  
nucleotides which are used in circular oligonucleotide  
synthesis. Commonly used enzymes include horseradish  
peroxidase, alkaline phosphatase, glucose oxidase and  $\beta$ -  
galactosidase, among others. The substrates to be used  
20 with the specific enzymes are generally chosen because a  
detectably colored product is formed by the enzyme  
acting upon the substrate. For example, p-nitrophenyl  
phosphate is suitable for use with alkaline phosphatase  
conjugates; for horseradish peroxidase, 1,2-  
25 phenylenediamine, 5-aminosalicylic acid or toluidine  
are commonly used. The probes so generated have utility  
in the detection of a specific DNA or RNA target in, for  
example, Southern analysis, Northern analysis, in situ  
hybridization to tissue sections or chromosomal squashes  
30 and other analytical and diagnostic procedures. The  
methods of using such hybridization probes are well

1 known and some examples of such methodology are provided  
by Sambrook et al.

The present circular oligonucleotides can be  
used in conjunction with any known detection or  
5 diagnostic procedure which is based upon hybridization  
of a probe to a target nucleic acid. Moreover, the  
present circular oligonucleotides can be used in any  
hybridization procedure which quantitates a target  
nucleic acid, e.g., by competitive hybridization between  
10 a target nucleic acid present in a sample and a labeled  
tracer target for one of the present oligonucleotides.

Furthermore, the reagents needed for making a circular  
oligonucleotide probe and for utilizing such a probe in  
a hybridization procedure can be marketed in a kit.

15 The kit can be compartmentalized for ease of  
utility and can contain at least one first container  
providing reagents for making a precircle precursor for  
a circular oligonucleotide, at least one second  
container providing reagents for labeling the precircle  
20 with a reporter molecule, at least one third container  
providing reagents for circularizing the precircle, and  
at least one fourth container providing reagents for  
isolating the labeled circular oligonucleotide.

Moreover the present invention provides a kit  
25 for isolation of a template nucleic acid. Such a kit  
has at least one first container providing a circular  
oligonucleotide which is complementary to a target  
contained within the template. For example, the  
template nucleic acid can be cellular and/or viral  
30 poly(A)<sup>+</sup> mRNA and the target can be the poly(A)<sup>+</sup> tail.  
Hence circular oligonucleotides of the present invention

1 which have utility for isolation of poly(A)+ mRNA have P  
and AP domain sequences of poly(dT) or poly(U).

Furthermore, the present invention provides  
kits useful when diagnosis of a disease depends upon  
5 detection of a specific, known target nucleic acid.  
Such nucleic acid targets can be, for example, a viral  
nucleic acid, an extra or missing chromosome or gene, a  
mutant cellular gene or chromosome, an aberrantly  
expressed RNA and others. The kits can be  
10 compartmentalized to contain at least one first  
container providing a circular oligonucleotide linked to  
a reporter molecule and at least one second container  
providing reagents for detection of the reporter  
molecule.

15 One aspect of the present invention provides a  
method of regulating biosynthesis of a DNA, an RNA or a  
protein by contacting at least one of the subject  
circular oligonucleotides with a nucleic acid template  
for that DNA, that RNA or that protein in an amount and  
20 under conditions sufficient to permit the binding of the  
oligonucleotide(s) to a target sequence contained in the  
template. The binding between the oligonucleotide(s)  
and the target blocks access to the template, and  
thereby regulates biosynthesis of the nucleic acid or  
25 the protein. Blocking access to the template prevents  
proteins and nucleic acids involved in the biosynthetic  
process from binding to the template, from moving along  
the template, or from recognizing signals encoded within  
the template. Alternatively, when the template is RNA,  
30 regulation can be accomplished by allowing selective  
degradation of the template. For example, RNA templates  
bound by the subject circular oligonucleotides are

1 susceptible to degradation by RNase H and RNase H  
degradation of a selected RNA template can thereby  
regulate use of the template in biosynthetic processes.

As used herein, biosynthesis of a nucleic acid  
5 or a protein includes cellular and viral processes such  
as DNA replication, DNA reverse transcription, RNA  
transcription, RNA splicing, RNA polyadenylation, RNA  
translocation and protein translation, and of which can  
lead to production of DNA, RNA or protein, and involve a  
10 nucleic acid template at some stage of the biosynthetic  
process.

As used herein, regulating biosynthesis  
includes inhibiting, stopping, increasing, accelerating  
or delaying biosynthesis. Regulation may be direct or  
15 indirect, i.e. biosynthesis of a DNA, RNA or protein may  
be regulated directly by binding a circular  
oligonucleotide to the template for that DNA, RNA or  
protein; alternatively, biosynthesis may be regulated  
indirectly by oligonucleotide binding to a second  
20 template encoding a protein that plays a role in  
regulating the biosynthesis of the first DNA, RNA or  
protein.

The nucleic acid templates can be RNA or DNA  
and can be single-stranded or double-stranded. While  
25 the present circular oligonucleotides bind to only one  
strand of a target present in the template, double-  
stranded templates are opened during biosynthetic  
processes and thereby become available for binding.  
Furthermore, the P domain of the present circular  
30 oligonucleotides can bind to a double-stranded target  
and place AP domain nucleotides in a position to compete  
for Watson-Crick binding to target nucleotides.



1 DNA replication from a DNA template is  
mediated by proteins which bind to an origin of  
replication where they open the DNA and initiate DNA  
synthesis along the DNA template. To inhibit DNA  
5 replication in accordance with the present invention,  
circular oligonucleotides are selected which bind to one  
or more targets in an origin of replication. Such  
binding blocks template access to proteins involved in  
DNA replication. Therefore initiation and procession of  
10 DNA replication is inhibited. As an alternative method  
of inhibiting DNA replication, expression of the  
proteins which mediate DNA replication can be inhibited  
at, for example, the transcriptional or translational  
level.

15 DNA replication from an RNA template is  
mediated by reverse transcriptase binding to a region of  
RNA also bound by a nucleic acid primer. To inhibit DNA  
replication from an RNA template, reverse transcriptase  
or primer binding can be blocked by binding a circular  
20 oligonucleotide to the primer binding site, and thereby  
blocking access to that site. Moreover, inhibition of  
DNA replication can occur by binding a circular  
oligonucleotide to a site residing in the RNA template  
since such binding can block access to that site and to  
25 downstream sites, i.e. sites on the 3' side of the  
target or binding site.

To initiate RNA transcription, RNA polymerase  
recognizes and binds to specific start sequences, or  
promoters, on a DNA template. Binding of RNA polymerase  
30 opens the DNA template. There are also additional  
transcriptional regulatory elements that play a role in  
transcription and are located on the DNA template.

1 These transcriptional regulatory elements include  
enhancer sequences, upstream activating sequences,  
repressor binding sites and others. All such promoter  
and transcriptional regulatory elements, singly or in  
5 combination, are targets for the subject circular  
oligonucleotides. Oligonucleotide binding to these  
sites can block RNA polymerase and transcription factors  
from gaining access to the template and thereby  
regulating, e.g., increasing or decreasing, the  
10 production of RNA, especially mRNA and tRNA.  
Additionally, the subject oligonucleotides can be  
~~targeted to the coding region or 3'-untranslated region~~  
of the DNA template to cause premature termination of  
transcription. One skilled in the art can readily  
15 design oligonucleotides for the above target sequences  
from the known sequence of these regulatory elements,  
from coding region sequences, and from consensus  
sequences.

RNA transcription can be increased by, for  
20 example, binding a circular oligonucleotide to a  
negative transcriptional regulatory element or by  
inhibiting biosynthesis of a protein that can repress  
transcription. Negative transcriptional regulatory  
elements include repressor sites or operator sites,  
25 ~~wherein a repressor protein binds and blocks~~  
transcription. Oligonucleotide binding to repressor or  
operator sites can block access of repressor proteins to  
their binding sites and thereby increase transcription.

The primary RNA transcript made in eukaryotic  
30 cells, or pre-mRNA, is subject to a number of  
maturation processes before being translocated into the  
cytoplasm for protein translation. In the nucleus,

1 introns are removed from the pre-mRNA in splicing  
reactions. The 5' end of the mRNA is modified to form  
the 5' cap structure, thereby stabilizing the mRNA.  
Various bases are also altered. The polyadenylation of  
5 the mRNA at the 3' end is thought to be linked with  
export from the nucleus. The subject circular  
oligonucleotides can be used to block any of these  
processes.

A pre-mRNA template is spliced in the nucleus  
10 by ribonucleoproteins which bind to splice junctions and  
intron branch point sequences in the pre-mRNA.  
Consensus sequences for 5' and 3' splice junctions and  
for the intron branch point are known. For example,  
inhibition of ribonucleoprotein binding to the splice  
15 junctions or inhibition of covalent linkage of the 5'  
end of the intron to the intron branch point can block  
splicing. Maturation of a pre-mRNA template can,  
therefore, be blocked by preventing access to these  
sites, i.e. by binding circular oligonucleotides of this  
20 invention to a 5' splice junction, an intron branch  
point or a 3' splice junction. Splicing of a specific  
pre-mRNA template can be inhibited by using circular  
oligonucleotides with sequences that are complementary  
to the specific pre-mRNA splice junction(s) or intron  
25 branch point. In a further embodiment, a collection of  
related splicing of pre-mRNA templates can be inhibited  
by using a mixture of circular oligonucleotides having a  
variety of sequences that, taken together, are  
complementary to the desired group of splice junction  
30 and intron branch point sequences.

Polyadenylation involves recognition and  
cleavage of a pre-mRNA by a specific RNA endonuclease at

1 specific polyadenylation sites, followed by addition of  
a poly(A) tail onto the 3' end of the pre-mRNA. Hence,  
any of these steps can be inhibited by binding the  
subject oligonucleotides to the appropriate site.

5 RNA translocation from the nucleus to the  
cytoplasm of eukaryotic cells appears to require a  
poly(A) tail. Thus, a circular oligonucleotide is  
designed in accordance with this invention to bind to  
the poly(A) tail and thereby block access to the poly  
10 (A) tail and inhibit RNA translocation. For such an  
oligonucleotide, both the P and AP domains can consist  
of about 10 to about 50 thymine residues, and preferably  
about 20 residues. Especially preferred P and AP domain  
lengths for such an oligonucleotide are about 6 to about  
15 12 thymine residues.

Protein biosynthesis begins with the binding  
of ribosomes to an mRNA template, followed by initiation  
and elongation of the amino acid chain via translational  
"reading" of the mRNA. Protein biosynthesis, or  
20 translation, can thus be blocked or inhibited by  
blocking access to the template using the subject  
circular oligonucleotides to bind to targets in the  
template mRNA. Such targets contemplated by this  
invention include the ribosome binding site (Shine-  
25 Delgarno sequence), the 5' mRNA cap site, the initiation  
codon, and sites in the protein coding sequence. There  
are also classes of protein which share domains of  
nucleotide sequence homology. Thus, inhibition of  
protein biosynthesis for such a class can be  
30 accomplished by targeting the homologous protein domains  
(via the coding sequence) with the subject circular  
oligonucleotides.

1           R gulation of biosynthesis by any of the  
aforementioned procedures has utility for many  
applications. For example, genetic disorders can be  
corrected by inhibiting the production of mutant or  
5 over-produced proteins, or by increasing production of  
an under-expressed proteins; the expression of genes  
encoding factors that regulate cell proliferation can be  
inhibited to control the spread of cancer; and virally  
encoded functions can be inhibited to combat viral  
10 infection.

Some types of genetic disorders that can be  
treated by the circular oligonucleotides of the present  
invention include Alzheimer's disease, some types of  
arthritis, sickle cell anemia and others. Many types of  
15 viral infections can be treated by utilizing the  
circular oligonucleotides of the present invention,  
including infections caused by influenza, rhinovirus,  
HIV, herpes simplex, papilloma virus, cytomegalovirus,  
Epstein-Barr virus, adenovirus, vesticular stomatitis  
20 virus, rotavirus and respiratory syncytial virus among  
others. According to the present invention, animal and  
plant viral infections may also be treated by  
administering the subject oligonucleotides.

The c-myc gene is one example of a gene which  
25 can have a role in cell proliferation. Inhibition of c-  
myc expression has been demonstrated in vitro using a  
linear oligonucleotide complementary to a target 115 bp  
upstream of the c-myc transcription start site (Cooney  
et al., 1988, Science 241: 456-459). Circular  
30 oligonucleotides of SEQ ID NO:1, and SEQ ID NO:2, as  
depicted below, are complementary to the c-myc promoter  
at nucleotides -131 to -120 and -75 to -62,

1 respectively, and are provided to inhibit c-myc  
expression in accordance with the present invention. As  
used in these depictions of SEQ ID NO:1 and SEQ ID NO:2,  
N can be any nucleotide or nucleotide analog.

5 SEQ ID NO:1

10

1  
N C T C C C C G C C C T C N  
N  
N  
N  
N C T C C C C A C C C T C N

SEQ ID NO:2

15

1  
N T C T T T T T C T T T C N  
N  
N  
N  
N T C T T T T T C T T T C N  
N

Human immunodeficiency virus (HIV) is a retrovirus causing acquired immunodeficiency syndrome (AIDS). The circular oligonucleotides of this invention provide a means of blocking the replication of the virus without deleteriously affecting normal cellular replication in humans infected with HIV. The retroviral genome is transcribed as a single, long transcript, part of which is spliced to yield RNA encoding viral envelope proteins. Inhibition of HIV infection can be accomplished by designing oligonucleotides to bind to a number of regions within the HIV genome, including coding regions for functions that replicate the genome (i.e., the pol or reverse transcriptase function) or functions that control gene expression (e.g. the tat,

1 rev or other functions). However, previous work with  
 linear oligonucleotides has suggested that splice sites,  
 poly(A) addition signals, cap or initiator codon sites,  
 and sites implicated in ribosome assembly can be  
 5 particularly effective for inhibiting eucaryotic protein  
 expression. Furthermore, the terminal structures of the  
 retroviral genome are also excellent targets for  
 inhibiting retrovirus production not only because these  
 structures encode control regions which mediate the rate  
 10 of transcription and replication, but also because these  
 structures are repeated, allowing an oligonucleotide to  
 bind and block access to each repeat.

Accordingly, the present invention provides  
 two circular oligonucleotides, set forth in SEQ ID NO:3  
 15 and SEQ ID NO:4 wherein N is any nucleotide or  
 nucleotide analog and Y is a pyrimidine or a pyrimidine  
 analog. SEQ ID NO:3 is complementary to an HIV-1 splice  
 junction (nucleotides 6039-52), while SEQ ID NO:4 is  
 complementary to part of the tat gene (nucleotides 5974-  
 20 88). The circular form of SEQ ID NO:3 is depicted  
 below, wherein nucleotide number 1 is the first  
 nucleotide in the P domain, i.e., the first T on the top  
 line corresponds to base 1.

25

```

      1
      N T T T C Y T C G T T C G T C N
    N
  N
  N
      N T T T C G T C A T T C A T C N
    N
  N
  N
  
```

The circular form of SEQ ID NO:4 is depicted below  
 30 wherein nucleotide number 1 is the first nucleotide of  
 the P domain.

35

1  
1  
N T C C T T C T T C Y C C T C T N  
N  
N  
N  
N  
5  
N T C C T T C T T C G C C T C T N

Circular oligonucleotides of SEQ ID NO:3 and SEQ ID NO:4 can inhibit HIV infection both in vitro and in vivo. In vitro screening for circular oligonucleotide effectiveness against HIV infection permits one skilled in the art to judge the stability of oligonucleotide: target binding and to assess in vivo efficacy and binding stability. To observe in vitro inhibition circular oligonucleotides can be added to the growth medium of an appropriate cell line infected with HIV. Cells can be pretreated with the circular oligonucleotides or circular oligonucleotides can be added at the time of infection or after HIV infection. Addition before or after infection allows assessment of whether the subject oligonucleotide can prevent or simply inhibit HIV infection respectively.

The extent of inhibition of HIV infection or replication can be judged by any of several assay systems, including assessment of the proportion of oligonucleotide-treated cells surviving after infection relative to survival of untreated cells, assessment of the number of syncytia formed in treated and untreated HIV infected cells and determination of the amount of viral antigen produced in treated and untreated cells.

30 In vivo studies of the efficacy of circular oligonucleotides can be done in a suitable animal host, such as transgenic mice, or chimpanzees. Levels of HIV



1 antigens can be monitored to assess the effect of  
circular oligonucleotides on HIV replication and thereby  
to follow the course of the disease state.

Alternatively, human volunteers with AIDS or ARC can be  
5 administered with the subject circular oligonucleotides  
since the oligonucleotides do not appear to be  
cytotoxic. The disease status of these volunteers can  
then be assessed to determine the efficacy of the  
subject oligonucleotides in treating and preventing AIDS  
10 infection.

A further aspect of this invention provides  
pharmaceutical compositions containing the subject  
circular oligonucleotides with a pharmaceutically  
acceptable carrier. In particular, the subject  
15 oligonucleotides are provided in a therapeutically  
effective amount of about 0.1  $\mu$ g to about 100 mg per kg  
of body weight per day, and preferably of about 0.1  $\mu$ g  
to about 10 mg per kg of body weight per day, to bind to  
a nucleic acid in accordance with the methods of this  
20 invention. Dosages can be readily determined by one of  
ordinary skill in the art and formulated into the  
subject pharmaceutical compositions.

As used herein, "pharmaceutically acceptable  
carrier" includes any and all solvents, dispersion  
25 media, coatings, antibacterial and antifungal agents,  
isotonic and absorption delaying agents, and the like.  
The use of such media and agents for pharmaceutical  
active substances is well known in the art. Except  
insofar as any conventional media or agent is  
30 incompatible with the active ingredient, its use in the  
therapeutic compositions is contemplated. Supplementary

1 active ingredients can also be incorporated into the compositions.

The subject oligonucleotides may be administered topically or parenterally by, for example, 5 intravenous, intramuscular, intraperitoneal subcutaneous or intradermal route, or when suitably protected, the subject oligonucleotides may be orally administered. The subject oligonucleotides may be incorporated into a cream, solution or suspension for 10 topical administration. For oral administration, oligonucleotides may be protected by enclosure in a ~~gelatin capsule.~~ Oligonucleotides may be incorporated into liposomes or liposomes modified with polyethylene glycol for parenteral administration. Incorporation of 15 additional substances into the liposome, for example, antibodies reactive against membrane proteins found on specific target cells, can help target the oligonucleotides to specific cell types.

Topical administration and parenteral 20 administration in a liposomal carrier is preferred.

The following examples further illustrate the invention.

25

30

35

1

EXAMPLE 1Circularization of Oligonucleotides Using an  
End Joining Oligonucleotide

5 According to the present invention, a simple one-step chemical method has been developed to construct circles from linear precursors (precircles). A DNA oligonucleotide was constructed which had the same sequence as the eventual target, this is the end-  
10 joining-oligonucleotide. A precircle oligonucleotide was then constructed and chemically phosphorylated on the 5'-end or 3'-end. As depicted in Fig. 2, the precircle and end-joining-oligonucleotide were mixed and allowed to form a complex in which the ends were  
15 adjacent. Cyanogen bromide, imidazole buffer, and a divalent metal were added. After incubation for 6-48 hr, the mixture was dialyzed, lyophilized, and the products were separated by denaturing 20% polyacrylamide gel electrophoresis. UV shadowing revealed major bands  
20 which comigrated with the precircle and the end-joining-oligonucleotide, along with one new product which migrated slightly more slowly than the precircle. No product was observed without added end-joining-oligonucleotide or in the absence of a 5'- or 3'-  
25 phosphate group on the precircle. The major bands were excised and eluted from the gel, dialyzed to remove salts and quantitated by absorbance at 260 nm. For reactions with precircles 1 and 2 (SEQ ID NO: 5 and SEQ ID NO: 6, respectively), using end-joining-  
30 oligonucleotides 4 and 5 (SEQ ID NO: 8 and SEQ ID NO: 9, respectively), the circles 6 and 7 were obtained in 40% and 58% yields, respectively. The sequences of each of

35

1 these molecules and other oligonucleotides are depicted  
in Fig. 3.

The circular structure of products 6 and 7 was confirmed by resistance to 3' exonuclease digestion and 5 to 5' dephosphorylation under reaction conditions in which a linear precircle was completely destroyed or dephosphorylated. Accordingly, the 3' exonuclease activity of T4 DNA polymerase cleaved linear precircles 1 and 2, but not circles 6 and 7. The linear precircles 10 were also 5'-end labeled with  $^{32}\text{P}$  and then circularized. After reaction, the circular products were inert to calf alkaline phosphatase whereas the precircles completely released labeled  $^{32}\text{P}$ . The slightly slower gel mobility of the circles relative to the precircles was consistent 15 with the occurrence of circularization.

#### Optimal Circularization Conditions

Many parameters were optimized to increase yields of the circular product, including oligonucleotide and precircle concentrations, 20 temperature, reaction time, metal, metal concentration, BrCN concentration and pH. Improved circularization conditions provided an at least two-fold higher yield of circles compared to prior art conditions wherein two single-stranded oligonucleotides were joined (Luebke 25 et al., 1989, J. Am. Chem. Soc. 111: 8733 and Kanaya et al., 1986, Biochemistry 25: 7423).

30

35

1 These improved conditions were:

- 50  $\mu$ M precircle
- 55  $\mu$ M end-joining-oligonucleotide
- 100 mM  $\text{NiCl}_2$
- 5 200 mM imidazole HCl (pH 7.0)
- 125 mM BrCN
- 25°C, 36 hr.

However circle closure was also effective under the following conditions:

- 10 3-200  $\mu$ M precircle
- 3-200  $\mu$ M end-joining-oligonucleotide
- 10-500 mM  $\text{NiCl}_2$
- 50-500 mM imidazole-HCl
- 20-200 mM BrCN
- 15 4-37°C, 6-48 hr.

Other metals ( $\text{Zn}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ) also work in place of  $\text{Ni}^{2+}$ . Additionally, the reaction is pH sensitive.

#### Closure in AP and P Domains

- 20 Closure of a circle in the AP domain was superior to closure in the P domain. Comparison of the circularization of precircles 2 and 3 (SEQ ID NO: 6 and SEQ ID NO: 7, respectively) around the same end-joining-oligonucleotide (i.e. 5, SEQ ID NO: 9) indicated that
- 25 circle 7 (having SEQ ID NO: 6) was formed with a 58% yield when closed in the AP domain (i.e. using precircle 2) and only a 35% yield when closed in the P domain (i.e. using precircle 3).

#### Condensing Reagents

- 30 Two reagents have been commonly used for chemical ligation of DNA and RNA, BrCN/imidazole/ $\text{NiCl}_2$  and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)

1 (Kanaya et al. 1986 Biochemistry 25: 7423 and Ashley et  
2 al. 1991 Biochemistry 30: 2927). Therefore, these  
3 reagents were directly compared for efficacy in  
4 ligating a precircle to circular oligonucleotide 6 (Fig.  
5 3 and SEQ ID NO: 5) using a dA<sub>12</sub> (SEQ ID NO: 8) end-  
6 joining-oligonucleotide.

7 BrCN/imidazole/NiCl<sub>2</sub> was used under the  
8 established optimal conditions except that ligation  
9 efficiency was observed at both 4°C and 25°C. EDC was  
10 used at 200 mM with 20 mM MgCl<sub>2</sub>, 50 mM MES (pH 6.0) at  
11 4°C or 25°C with incubation for 4 days.

12 At 4°C BrCN was more efficient, yielding 95%  
13 circular product while EDC yielded only 55% product.  
14 However, at 25°C both EDC and BrCN yielded 95% product.  
15 Therefore, BrCN is more effective at lower temperatures  
16 but either EDC or BrCN can be used with equal success at  
17 25°C. However, BrCN has an additional advantage over  
18 EDC since ligation with BrCN requires 24 hr or less  
19 while ligation with EDC requires about 4 days.

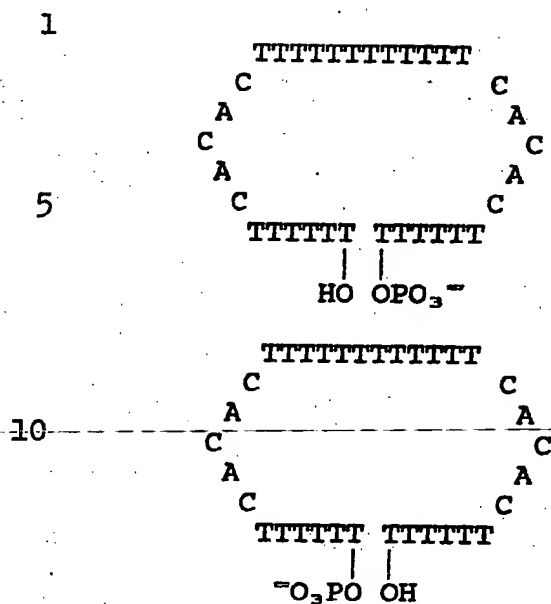
## 20 Use of a 5'- or 3'-Phosphate

21 Under different ligation conditions joining a  
22 3'-phosphate with a 5'-OH yielded more ligated product  
23 than joining a 5'-phosphate with a 3'-OH (Ashley, et  
24 al.).

25 Therefore, the percent conversion to circular  
26 oligonucleotide 6 (Fig. 3) by a 5'-phosphate or by a 3'-  
27 phosphate precircles was compared:

30

35



15 Circularization reactions were performed using  
a dA<sub>12</sub> end-joining-oligonucleotide and the established  
optimal conditions, except that 5 nmoles of precircle  
and end-joining-oligonucleotide were used. Products  
were visualized under UV light after separation by  
20 denaturing gel electrophoresis.

Conversion to circular product was 60% ( $\pm 5\%$ ) when a 5'-phosphate was present and 95% when a 3'-phosphate was present. No increase in yield was observed when increased reaction times or increased reagent concentrations were used.

Accordingly, use of a 3'-phosphate rather than a 5'-phosphate improves circularization.

1

EXAMPLE 2Circular Oligonucleotides Bind Target  
Nucleic Acids with Higher Affinity  
Than Do Linear Oligonucleotides

5 The binding affinities of circles 6 and 7 (SEQ  
ID NO: 5 and SEQ ID NO: 6, respectively) for their  
targets were measured by comparison of the melting  
temperatures of the circular and linear complexes.  
Solutions contained 1:1 ratios of oligonucleotide and  
10 target (3  $\mu\text{M}$  each) in 100 mM NaCl, 10 mM  $\text{MgCl}_2$ , and 10  
mM Tris-HCl (pH 7.0). Mixing curves measured at 260 nm  
confirmed that 1:1 complexes were formed. The free  
energies ( $-\Delta G^\circ_{37}$ ) of the complexes were derived from  
the melting data using a two-state curve-fitting method  
(Petersheim, et al., 1983, Biochemistry 22: 256).

15

The results showed that the circular  
oligonucleotides bound to their targets more strongly  
than did linear precircles or Watson-Crick complementary  
target-sized oligonucleotides (Table 2). For example,  
target 4 (SEQ ID NO: 8) formed a duplex with its target-  
20 sized Watson-Crick complement having a  $T_m$  of 37.1°C  
while the precircle 1:target 4 complex (i.e. SEQ ID NO:  
5 bound to SEQ ID NO: 8) had a  $T_m$  of 44.7°C. By  
comparison, circle 6, having the same sequence as  
precircle 1, bound to target 4 with a  $T_m$  of 57.5°C and a  
25 free energy of binding that was 8.6 kcal/mol more  
favorable than the corresponding Watson-Crick duplex.  
The corresponding association constant at 37°C is  $6 \times 10^{11} \text{ M}^{-1}$ , which is more than six orders of magnitude  
greater than for the Watson-Crick duplex. A similar  
30 effect was observed for the binding of circle 7 (SEQ ID  
NO: 6) to target 5 (SEQ ID NO: 9); this complex had a  $T_m$

35



1 of 62.3°C, whereas the corresponding Watson-Crick duplex  
melted at 43.8°C. These data indicate that the binding  
of circular oligonucleotides is stronger than the  
binding of a linear oligonucleotide to a target.

5 To determine the binding characteristics when  
the target sequence was embedded within a longer  
sequence, a 36 nucleotide oligonucleotide was  
synthesized with a 12 base target sequence (equivalent  
to target 4) in the middle. Melting studies revealed  
10 that circle 6 bound to this longer oligonucleotide more  
~~strongly than it did to a target having the same size as~~  
the binding domains of the circle: the  $T_m$  of circle 6  
with target 4 was 59.8°C whereas with the 36 base  
oligonucleotide containing an embedded target the  $T_m$  was  
15 63.4°C. Therefore the binding strength of circles with  
embedded targets was higher than that with binding-  
domain-sized-targets.

The binding affinity of circle 6 for an RNA  
target was tested by synthesizing oligoribonucleotide  
20  $rA_{12}$  and determining the  $T_m$  of circle 6 with  $rA_{12}$ . The  
 $T_m$  of circle 6 with  $rA_{12}$  was 58.3°C compared with 57.8°C  
with  $dA_{12}$ . The data indicate that circles bind to RNA  
targets as strongly or more strongly than as to DNA  
targets.

25

30

35

-59-

1

TABLE II

| 5  | oligonucleotide: |  | $T_m, ^\circ\text{C}$ | - $G^\circ_{37}(\text{kcal/mol})$ |
|----|------------------|--|-----------------------|-----------------------------------|
|    | target           | complex  |                       |                                   |
| 10 |                  | 3'-TTTTTTTTTTTT<br>5'-AAAAAAAAAAAA   | 37.1                  | 8.1                               |
|    |                  | 3'-TTCTTTTCTTTC<br>5'-AAGAAAAGAAAG   | 43.8                  | 10.3                              |
| 15 | 1:4              | <pre> TTTTTTTTTTTT   C             C   A             A C  AAAAAA      C   A             A   C             C   TTTTTT TTTTTT         OPO<sub>3</sub><sup>-</sup> </pre> | 44.7                  | 10.5                              |
| 20 | 3:5              | <pre> TTCTTTTCTTTC   C             C   A             A C  AAGAAA      C   A             A   C             C   TTCTTT TCTTTC         OPO<sub>3</sub><sup>-</sup> </pre> | 47.0                  | 10.8                              |
| 25 |                  |  |                       |                                   |
| 30 | 6:4              | <pre> TTTTTTTTTTTT   C             C   A             A C  AAAAAA      C   A             A   C             C   TTTTTT TTTTTT         OPO<sub>3</sub><sup>-</sup> </pre> | 57.4                  | 16.7                              |
| 35 | 7:5              | <pre> TTCTTTTCTTTC   C             C   A             A C  AAGAAA      C   A             A   C             C   TTCTTTTCTTTC         OPO<sub>3</sub><sup>-</sup> </pre>  | 62.3                  | 16.4                              |

-60-

1

EXAMPLE 3  
Circular Oligonucleotides Bind Target More  
Selectively Than Linear Oligonucleotides

5           In order to measure the sequence selectivity of  
circular oligonucleotides, a set of target  
oligonucleotides with one variable base was constructed.  
Binding energies for a circle complexed with these  
targets were measured; the selectivity was defined by the  
10 free energy difference between the correct sequence and  
mismatched sequences. The selectivity obtained with the  
circular structure was then directly compared to the  
selectivity of an analogous linear oligonucleotide.

          DNA oligonucleotides were machine synthesized  
15 using the 8-cyanoethyl phosphoramidite method. Circular  
oligonucleotide 8 was prepared from a linear precircle  
having SEQ ID NO: 7:

          5'-pTCTTTCCACACCTTTCTTTCTTCACACTTCTTT  
and was cyclized by assembly around an end-joining  
20 oligonucleotide having the sequence 5'-AAGAAAAGAAAG (SEQ  
ID NO: 9) using BrCN/imidazole to close the final bond,  
as described in Example 1. The circular structure was  
confirmed by its resistance to a 3'-exonuclease and 5'-  
phosphatase.

25           The sequence selectivity of circle 8 was  
measured by hybridizing it with targets which contained a  
single mismatched base and determining the strength  
( $\Delta G^\circ_{37}$ ) of the resulting complexes by thermal  
denaturation. Eight targets were synthesized which were  
30 complementary to circle 8 and linear oligonucleotide 9  
except for a single centrally positioned variable base (X

35

-61-

1

or Y = A, G, C, T). Four targets have a variable base X which is matched with two opposing T's in the circle, resulting in a T-X-T triad. In the remaining four

5 targets, the variable base Y is matched with two opposing C's in the circle, giving a C-Y-C triad. For comparison to this circle complex, a linear oligonucleotide 9 was used; resulting in a duplex with a central T-X pair in the first four experiments or a C-Y pair in the remaining  
10 four.

|    | <u>complex (X,Y = A,T,G,C)</u>  | <u>expt. no.</u> |
|----|---------------------------------|------------------|
|    | 3' - T T C T T T T C T T T C    |                  |
|    | 5' - A A G A X A A G A A A G    | 1-4              |
| 15 | A C T T C T T T T C T T T C C A |                  |
|    | C A A G A X A A G A A A G C     | 5-8              |
|    | A C T T C T T T T C T T T C C A |                  |
| 20 | 3' - T T C T T T T C T T T C    |                  |
|    | 5' - A A G A A A A Y A A A G    | 9-12             |
|    | A C T T C T T T T C T T T C C A |                  |
| 25 | C A A G A A A A Y A A A G C     | 13-16            |
|    | A C T T C T T T T C T T T C C A |                  |

Thermal denaturation of the sixteen complexes was carried out in the presence of 10 mM MgCl<sub>2</sub>, 100 mM NaCl, and 10 mM Tris-HCl (pH 7.0), with target and  
30 circular or linear oligonucleotide concentrations at 3 μM

35

-62-

1

each. Experiments were carried out in duplicate and the results averaged. Oligonucleotide:target complex melting was monitored at 260 nm. The temperature vs. absorbance curves so generated showed a single transition from bound to free oligonucleotide. Free energies of association were obtained by fitting the data with a two-state curve-fitting method. The results were checked in two cases by measuring the association energies by the van't Hoff method, good agreement was seen between the two methods.

10

Selectivities are defined as the difference in free energies ( $\Delta G$ ) of complexation between matched and mismatched oligomers.

Table III displays the results of the mismatch experiments. Experiments 1-4 show the effects of a T-X target mismatch on a DNA duplex. As expected, the true match ( $X = A$ ) gives the most favorable complex ( $-\Delta G^\circ_{37} = 10.3$  kcal/mol); the mismatches ( $X = G, C, T$ ) result in a loss of 3.2-4.4 kcal/mol in binding energy, in good agreement with published mismatch studies. Experiments 5-8, by comparison, show the effects of a T-X-T mismatch on circle complex binding strength. Once again, the true match ( $X = A$ ) gives the most favorable three stranded complexes ( $-\Delta G^\circ_{37} = 16.4$  kcal). However, target mismatches ( $X = G, T, C$ ) result in a considerably larger loss of binding energy (6.2-7.6 kcal/mol) for a circular oligonucleotide than for a linear oligonucleotide.

25

Similarly, experiments 9-12 give the effects of a C-Y mismatch on the two stranded duplex. The matched base ( $Y = G$ ) gives a free energy of duplex association of -10.3 kcal/mol. The mismatches ( $Y = A, T, C$ ) result in a

35

-63-

1

loss of 5.2 to 5.8 kcal/mol of binding energy, in reasonable agreement with published data. By contrast, the effects of a C-Y-C mismatch are greater in a three  
5 stranded complex (experiments 13-16): the match (Y = G) gives a binding energy of -16.4 kcal/mol, and the mismatches (Y = A, T, C) are less stable by 7.1 to 7.5 kcal/mol.

Thus, in all the cases studied, the circular  
10 ligand shows greater selectivity for its correctly matched sequence than does the standard linear oligomer.

The selectivity advantage ranges from 1.3 to 2.2 kcal/mol for the C-Y-C series to 3.0 to 3.4 kcal/mol for the T-X-T series. These are quite significant differences,  
15 considering they arise from a single base change; in the T-X-T series, the circular oligonucleotide is nearly twice as selective as the linear oligonucleotide. This selectivity difference corresponds to one to two orders of magnitude in binding constant at 37°C.

20 There are two factors which may explain this high selectivity. First, because two domains of the circular oligonucleotide bind the central target strand, the circular oligonucleotide, in effect, checks the sequence twice for correct matching. Secondly,

25 protonation of cytosine within a C+G-C triad may also be a factor in increasing selectivity. This protonation is likely to be favored only when there is base triad formation wherein guanine can share the positive charge; evidence suggests that the pKa of cytosine within a base  
30 triad is 2-3 units higher than that of free deoxycytosine. The addition of this positive charge may

35

-64-

1

lessen the negative charge repulsions arising from the high density of phosphates in the complex and thereby increase binding stability.

5

Therefore, circular oligonucleotides, as described herein, to have both higher binding affinity and higher selectivity than can be achieved with Watson-Crick duplexes alone.

10

15

20

25

30

35

-65-

1

TABLE III

| 5  |                   | expt. # | variable<br>base | $T_m, ^\circ\text{C}$ | $-\Delta G^\circ_{37}$<br>(kcal/mol) | Selectivity<br>(kcal/mol) |
|----|-------------------|---------|------------------|-----------------------|--------------------------------------|---------------------------|
|    |                   |         |                  |                       |                                      |                           |
| 10 | duplex            | 1       | X=A              | 43.8                  | 10.3                                 | --                        |
|    |                   | 2       | X=G              | 33.8                  | 7.1                                  | 3.2                       |
|    |                   | 3       | X=C              | 28.3                  | 5.9                                  | 4.4                       |
|    |                   | 4       | X=T              | 31.1                  | 6.4                                  | 3.9                       |
| 15 | circle<br>complex | 5       | X=A              | 62.3                  | 16.4                                 | --                        |
|    |                   | 6       | X=G              | 44.2                  | 10.2                                 | 6.2                       |
|    |                   | 7       | X=C              | 39.8                  | 8.8                                  | 7.6                       |
|    |                   | 8       | X=T              | 40.8                  | 9.1                                  | 7.3                       |
| 20 | duplex            | 9       | Y=A              | 26.2                  | 5.1                                  | 5.2                       |
|    |                   | 10      | Y=G              | 43.8                  | 10.3                                 | --                        |
|    |                   | 11      | Y=C              | 22.2                  | 4.5                                  | 5.8                       |
|    |                   | 12      | Y=T              | 27.0                  | 5.0                                  | 5.3                       |
| 25 | circle<br>complex | 13      | Y=A              | 39.9                  | 9.0                                  | 7.4                       |
|    |                   | 14      | Y=G              | 62.3                  | 16.4                                 | --                        |
|    |                   | 15      | Y=C              | 41.3                  | 9.3                                  | 7.1                       |
|    |                   | 16      | Y=T              | 39.6                  | 8.9                                  | 7.5                       |

30

35

SUBSTITUTE SHEET



-66-

1

EXAMPLE 4Factors Effecting Complex Formation

1) Solution effects. The effects of NaCl, Mg<sup>2+</sup>, spermine, and pH on circle:target complexes were examined. Circles with cytosines in the binding domains are sensitive to pH, and exhibited greater stability at lower pH values. However, these and other circle:target complexes are quite stable at the physiological pH of 7.0-7.4 (Fig. 5). The complexes show salt concentration sensitivity comparable to duplexes; however, small amounts of Mg<sup>2+</sup> or spermine increase the complex stability markedly. For example, in a concentration of 1 mM Mg<sup>++</sup> at pH 7.0, with no added salts, a stable 7:5 circle:target complex formed having a T<sub>m</sub> of 58°C. When a solution of 20 μm spermine containing no added salts was used the 7:5 complex again formed stably with a T<sub>m</sub> of 56°C. Both Mg<sup>++</sup> and spermine are present in at least these concentrations in mammals, and so circle:target complexes will be stable under physiological conditions.

2) Loop size. The optimum number of nucleotides for the loop domain of a circle was determined by observing complex formation between a target and circles with different loop sizes. Precircle linear oligonucleotides similar to precircle 1 were synthesized with 2, 3, 4, 5, 6 and 10 base loops using an arbitrary sequence of alternating C and A residues. Each of these precircles was designed to bind to the A<sub>12</sub> template (i.e. target 4 (SEQ ID NO: 8)). The T<sub>m</sub>'s for circles with 4, 5, 6 and 10 base loops showed that a five-nucleotide loop size was optimum for the circle

35

-67-

1

binding either to template  $A_{12}$  or to a longer 36mer sequence containing the  $A_{12}$  binding site (see Fig. 6A).

3) Binding Domain length. The effect of  
5 circular oligonucleotide binding domain length on circle:target complex melting temperature was compared to melting of duplexes having the same length. Circles with various size binding domains were constructed and complexed with single-stranded  $dA_n$  targets for  $n$  equal to  
10 4, 8, 12 and 18 nucleotides. Fig. 6B illustrates that considerably higher  $T_m$ 's were observed for circle:target complexes relative to Watson-Crick duplexes having the same length as the binding domains (determined in 0.1 M NaCl, pH 7). For example, a 12-base circular complex  
15 melted at about the same temperature as a 24-base duplex. The 4-base circular complex melted at 34°C, whereas the corresponding Watson-Crick duplex  $T_m$  was less than 0°C.

4) Methylation. It has been known for some time that methylation at the C-5 position of cytosine,  
20 forming the naturally-occurring base  $m^5C$ , raises the  $T_m$  of duplex DNA in which it occurs, relative to unmethylated sequences (Zmudzka et al., 1969, Biochemistry 8: 3049). In order to investigate whether addition of this methyl group would stabilize  
25 circle:target complexes, two analogs of circle 7 (having SEQ ID NO: 6) were synthesized. In one circle, the six C's in the binding domains were methylated leaving the loop unmethylated ( $Me_6$ ). In the second circle, all twelve C's were methylated ( $Me_{12}$ ). Melting temperatures  
30 for the complexes of these methylated circle with target 5 were measured. The  $Me_6$  complex had a  $T_m$  of 71.1°C

35

SUBSTITUTE SHEET

-68-

1

(compared to 61.8°C for the unmethylated circle), and the Me<sub>12</sub> circle had a T<sub>m</sub> of 72.4°C. Thus, use of the natural base m<sup>5</sup>C in place of C increased stability substantially, and in one case resulted in a 12-base complex which melted 10.6°C higher than an unmethylated circle and 28.6°C higher than the corresponding unmethylated Watson-Crick duplex.

10

15

20

25

30

35

-69-

1

**EXAMPLE 5****Replacement of Nucleotide Loop Domains  
with Non-Nucleotide Loop Domains**

5

The loop domains of circular oligonucleotides were replaced with polyethylene or oligoethylene glycol chains of different lengths and the effect of such synthetic loops upon circular oligonucleotide binding and nuclease resistance was assessed.

10

**Methods**

Circular oligonucleotides were synthesized having tetra-, penta-, or hexa-ethylene glycol chain loop domains. In each case the ethylene glycol chain was synthetically prepared for automated DNA synthetic procedures using the method of Durand *et al.* (1990, Nucleic Acids Res. 18: 6353-6359). Briefly, a phosphoramidite was placed on a hydroxy group at one end of the ethylene glycol chain and a dimethoxytrityl (DMT) moiety was placed on the other terminal ethylene glycol hydroxy group. This derivatized ethylene glycol chain was then added to the growing linear oligonucleotide at the appropriate step of automated DNA synthesis. Circularization steps were performed by procedures described in Example 1. A linear oligonucleotide precircle having a tetraethylene loop domain was not efficiently circularized. This result indicates that a tetraethylene loop domain may be too short for optimal binding to a target.

Two types of linear oligonucleotides were used as target binding domains for the circular oligonucleotides: Target I was a 12-base oligonucleotide

35

-70-

1

having no non-target nucleotides and Target II was a 36-base oligonucleotide having a 12-base target within it.

5 The target sequences utilized were 5'-AAGAAAAGAAAG-3' (SEQ ID NO: 9) and 5'-AAAAAAAAAAA-3' (SEQ ID NO: 8), the latter is termed a poly(dA)<sub>12</sub> target sequence.

The melting temperatures (T<sub>m</sub>) of circular oligonucleotides with polyethylene loops were observed at pH 7.0 (10 mM Tris-HCl) in 10 mM MgCl<sub>2</sub> and 100 mM NaCl.  
10 Each linear target and each circular oligonucleotide was present at a 3 μM concentration.

### Results

The T<sub>m</sub> of a circular oligonucleotide having a CACAC nucleotide loop sequence and a poly(dT)<sub>12</sub> sequence  
15 for both P and AP domains was 57.8°C when bound to a poly(dA)<sub>12</sub> target sequence. The T<sub>m</sub> of a circular oligonucleotide having the same P and AP domain sequences but hexaethylene glycol loop domains was 51.4 °C when bound to the same target.

20 A comparison of T<sub>m</sub> values observed for circular oligonucleotides having pentaethylene glycol (PEG) and hexaethylene glycol (HEG) loop domains is depicted in Table IV.

25

30

35

-71-

1

TABLE IV

| 5  | Complex                       | Target           |                   |
|----|-------------------------------|------------------|-------------------|
|    |                               | I T <sub>m</sub> | II T <sub>m</sub> |
| 10 | p T T C T T T T C T T T C p   |                  |                   |
|    | PEG A A G A A A G A A A G PEG | 51.5             | 47.5              |
| 15 | p T T C T T T T C T T T C p   |                  |                   |
|    | HEG A A G A A A G A A A G HEG | 58.0             | 51.1              |
| 20 | p T T T T T T T T T T T T p   |                  |                   |
|    | HEG A A A A A A A A A A A HEG | 51.4             | 46.5              |

The T<sub>m</sub> value observed for a circular oligonucleotide having a HEG loop is about 4.5°C higher than that of a circular oligonucleotide with a PEG loop. Therefore,

25 circular oligonucleotides with hexaethylene glycol loop domains bind with greater stability than do circular oligonucleotides with tetra- or penta-ethylene glycol loops.

30

35

-72-

1

Nuclease Resistance

5 Circular oligonucleotides were tested for  
nuclease resistance when unbound and when bound to a  
target oligonucleotide. All circular oligonucleotides,  
whether bound or unbound, were completely resistant to  
exonucleases. Endonuclease sensitivity was assessed  
using S1 nuclease according to the manufacturer's  
suggestions.

10

A comparison of the resistance of bound and  
unbound circular oligonucleotides to S1 nuclease is  
depicted in Table V.

15

20

25

30

35

-73-

1

TABLE V

| 5  | Oligonucleotide<br>Cleavage   | Time For<br>50% S1 |
|----|---|--------------------|
| 10 | P T T C T T T T C T T T C P<br>HEG HEG  | 1 min.             |
| 15 | P T T C T T T T C T T T C P<br>HEG A A G A A A G A A A G HEG<br>P T T C T T T T C T T T C P     | > 24 h             |
| 20 | A C T T C T T T T C T T T C C A<br>C C<br>A C T T C T T T T C T T T C C A                       | 1 min.             |
| 25 | A C T T C T T T T C T T T C C A<br>C A A G A A A G A A A G C<br>A C T T C T T T T C T T T C C A | 40 min.            |

30

35



-74-

1

These data indicate that unbound circular oligonucleotides are vulnerable to S1 nuclease. However, when bound to a target, a circular oligonucleotide having a polyethylene loop domain is much more resistant to S1 nuclease, at least 36-fold more resistant, than a circular oligonucleotide with a nucleotide loop domain.

The nuclease resistance of circular and linear oligonucleotides was also compared when these oligonucleotides were incubated in human plasma for varying time periods. Circular oligonucleotide 7 and the precursor to this circle, linear oligonucleotide 2, were incubated at a 50  $\mu$ M concentration in plasma at 37°C. Aliquots were removed at various time points and cleavage products were separated by gel electrophoresis. Nuclease resistance was assessed by observing whether degradation products were evident on the gels.

When incubated in human plasma the half-life of linear oligonucleotide 2 was 20 min. In contrast, circular oligonucleotide 7 underwent no measurable nuclease degradation during a 48 hr incubation. Accordingly, the half-life of a circular oligonucleotide is greater than 48 hr in human plasma, i.e. more than 140 times longer than a linear oligonucleotide having an equivalent sequence.

30

35

-75-

1

EXAMPLE 6CIRCULAR OLIGONUCLEOTIDES CAN SELECTIVELY  
BIND TO RNA

5

Experiments described in this Example indicate that, unlike linear oligonucleotides, circular oligonucleotides can preferentially bind to an RNA, rather than a DNA, target.

10

Two linear deoxyoligonucleotides were prepared as targets, a "T" (SEQ ID NO.: 11) target and a "dU" (SEQ. ID. No.: 12) target:

T target: 5'-A A G A A T A G A A A G-3'; and

15

dU target: 5'-A A G A A U A G A A A G-3'.

A circular oligonucleotide having SEQ ID NO.: 14 was also prepared:

20



For comparison, a linear oligonucleotide complementary to the T and dU targets was also synthesized (i.e. the linear oligonucleotide, SEQ ID NO.: 13):

25

5'C T T T C T A T T C T T 3'.

The melting temperatures ( $T_m$ ) values observed for the circular vs linear oligonucleotide binding to each of the targets is presented in Table VI.

30

35

-76-

1

TABLE VI

5

10

| Targets   | <u>T<sub>m</sub> Values for Oligonucleotides</u> |          |
|-----------|--|----------|
|           | Linear   | Circular |
| T target  | 42.9° C  | 41.1° C  |
| dU target | 40.9° C  | 42.9° C  |

The linear oligonucleotide binds more strongly to the T target than to the dU target, by an amount which is significantly larger than experimental error limits. This difference in T<sub>m</sub> values corresponds to a difference in free energy of binding of 1.7 kcal/mole.

However, in contrast to the linear oligonucleotide, the circular oligonucleotide binds more strongly to the U target. Therefore, the circular oligonucleotide can exhibit a preference for an RNA target relative to the corresponding DNA target.

Moreover, the increase in binding strength for a circular oligonucleotide to the RNA target corresponds to a free energy difference of 0.8 kcal/mole which indicates that at 37 °C an RNA target would be preferred by about 3:1 over a corresponding DNA target.

30

35

-77-

1

EXAMPLE 7Strand Replacement By Circular Oligonucleotides

5 Circular oligonucleotide 6 (Fig. 3) bound to a dA<sub>12</sub> target with 9 kcal/mole greater stability than did a linear dT<sub>12</sub> oligonucleotide (Example 2). This increase in stability demonstrates that a circular-oligonucleotide:target complex is thermodynamically  
10 favored over a linear-oligonucleotide:target. In addition, a circular oligonucleotide can actually accelerate (or catalyze) dissociation of duplex DNA target sequences to form a complex with one strand of the duplex.

15 To test whether a circular oligonucleotide can readily dissociate duplex DNA and displace one strand of a duplex DNA target, the kinetics of strand displacement were observed for a duplex DNA target in the presence of a complementary linear or circular oligonucleotide.

20 A DNA duplex target with a fluorescein group on one strand and a tetramethylrhodamine group on the other strand was prepared using published procedures (Cardullo et al. 1988 Proc. Natl. Acad. Sci. USA 85: 8790; Cooper et al. 1990 Biochemistry 29: 9261). The structure of the  
25 duplex target (SEQ ID NO.: 15) was as follows:

5'-fluorescein-A A A A A A A A A A A  
3'-rhodamine-T T T T T T T T T T T.

The T<sub>m</sub> of this labeled duplex target was normal, therefore the fluorescent substituents had no significant  
30 effect upon association kinetics. Moreover, the emission maxima of the fluorescein-dA<sub>12</sub> strand was 523 nm while the

35

-78-

1

emission maxima of the rhodamine-dT<sub>12</sub> strand was 590 nm, allowing the association kinetics of the two strands could be separately monitored.

5

10

Strand displacement reactions were done at 10°C in a 1 cm fluorescence cuvette. Reaction conditions were 100 mM NaCl, 10 mM Mg Cl<sub>2</sub> and 10 mM Tris-HCl, pH 7.0 with a reaction volume of 3 ml. Labeled duplex was allowed to equilibrate for at least 1 hr at 10°C before addition of a 40-fold excess of linear or circular oligonucleotide (final concentration 0.01 μM). A Spex Fluorolog F 111A fluorescence instrument with 5 mm slit widths was used. An excitation wavelength of 450 nm and a monitored emission wavelength of 523 nm was used. The results were independent of both excitation and monitored emission wavelengths. Reactions were followed for at least 5 half-lives.

15

Addition of rhodamine-dT<sub>12</sub> to fluorescein-dA<sub>12</sub> caused a decrease in fluorescein fluorescence and an increase in rhodamine fluorescence. Such effects are due to energy transfer between the fluorescent moieties (Cardullo et al.).

20

The association rate constant of the two fluorescently-labeled strands was determined by mixing the strands under pseudo-first order conditions and monitoring the rate of decrease in fluorescein emission. At 10 °C the observed association constant was  $3.2 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$ , which agrees well with published rates of association for DNA oligonucleotides (Nelson et al. 1982 Biochemistry 21: 5289; Turner et al. 1990 in Nucleic

25

30

-79-

1

Acids (subvolume C), W. Saenger, Ed. Springer-Verlag, Berlin: 201-227).

5 To compare the rates at which a single linear strand (SEQ ID NO.: 8) or a circular oligonucleotide having SEQ ID NO.: 5 (i.e. circular oligonucleotide 6) exchanged with strands in a duplex DNA, an excess of an unlabeled linear or circular oligonucleotide was mixed with the fluorescently-labeled duplex DNA target. The increase in fluorescein emission was then observed at a temperature significantly below the  $T_m$  of the duplex target as a measure of duplex target strand dissociation.

Fig. 8 depicts a typical kinetic assay for the dissociation of duplex target by a 40-fold excess of unlabeled  $dA_{12}$  (dotted line) or circular oligonucleotide 6 (solid line) at 10 °C. As depicted, duplex target dissociation by the circular oligonucleotide is considerably faster than is the dissociation by the linear oligonucleotide. The first order rate constant for dissociation by the linear oligonucleotide is  $2.0 \times 10^{-4} \text{ sec}^{-1}$  whereas the first order rate constant for dissociation by the circular oligonucleotide is  $2.3 \times 10^{-2} \text{ sec}^{-1}$ , almost two orders of magnitude faster. This difference is even more apparent when the half-lives for the target duplex in the presence of linear vs circular oligonucleotides are calculated. At 10 °C, the duplex has a half-life for dissociation of 58 min in the presence of the linear oligonucleotide but only 30 sec in the presence of the circular oligonucleotide.

Unlike the rate of reaction between linear oligonucleotide and duplex, the rate of reaction between

35

-80-

1

the circular oligonucleotide and duplex is dependent on the concentration of added circular oligonucleotide at low concentrations, and shows Michaelis-Menten type

5 saturation behavior at higher concentrations (Fig. 9).

The dissociation rate of labeled duplex at 10°C can be derived from the duplex association rate constant and  $\Delta G^\circ_{10}$  values. This rate constant,  $8.5 \times 10^{-10} \text{ sec}^{-1}$ , is consistent with rates derived from predicted

10 thermodynamic parameters for a duplex complex (Breslauer et al. 1986 Proc. Natl. Acad. Sci. USA 83: 3746) although this rate is significantly slower than the rate constant for strand displacement by a linear oligonucleotide. An increase in duplex dissociation upon addition of a linear  
15 oligonucleotide has been noted in other cases (Chamberlin et al. 1965 J. Mol. Biol. 12: 410). Comparison of the rate for the circular oligonucleotide-catalyzed reaction over that of the unassisted duplex dissociation reveals a rate enhancement of about  $10^7$  fold (Sigler et al. 1962 J.  
20 Mol. Biol. 5: 709) .

A double reciprocal plot of  $1/[\text{circular oligonucleotide}]$  vs.  $1/k_{\text{obs}}$  is linear and yields a  $k_{\text{cat}}$  of  $0.024 \pm 0.005 \text{ sec}^{-1}$  and a  $K_M$  of  $2.2 \times 10^{-7} \text{ M}$ . The  $k_{\text{cat}}$  is 100-fold greater than the observed rate constant  
25 obtained for the reaction of the duplex with either  $dA_{12}$  or  $dT_{12}$  single strands.

The observed saturation behavior (Fig. 9) suggests that a complex forms between the circle and the double-stranded target. Using the above  $K_M$  value and  
30 assuming that  $k_{\text{cat}} \ll k_{-1}$ , where  $k_{-1}$  is the dissociation rate constant for this complex, the free energy of

35

-81-

1

association is  $-8.6 \text{ kcal} \cdot \text{mol}^{-1}$  at  $10^\circ\text{C}$ . This value is similar to an estimated value of about  $-9 \text{ kcal} \cdot \text{mol}^{-1}$  for the P domain in a 12-base triple helix consisting of T·A-T base triads, as derived from the thermodynamic parameters of Pilch et al. (1990 Nucleic Acids Res. 18: 5743).

10

15

20

25

30

35



-82-

1

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

(i) APPLICANT: Kool, Eric T.

(ii) TITLE OF INVENTION: SINGLE-STRANDED, CIRCULAR  
OLIGONUCLEOTIDES

(iii) NUMBER OF SEQUENCES: 15

10

## (iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Scully, Scott, Murphy &amp; Presser

(B) STREET: 400 Garden City Plaza

(C) CITY: Garden City

(D) STATE: New York

(E) COUNTRY: USA

(F) ZIP: 11530

15

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version

#1.25

20

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US

(B) FILING DATE:

(C) CLASSIFICATION:

## (viii) ATTORNEY/AGENT INFORMATION:

25

(A) NAME: McNulty, William E.

(B) REGISTRATION NUMBER: 22,606

(C) REFERENCE/DOCKET NUMBER: 8085Z

## (ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (516) 742-4343

(B) TELEFAX: (516) 742-4366

## 30 (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

35

-83-

1

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: circular

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCCCCGCCC TCNNNNNCTC CCACCCCTCN NNNN  
34

10 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: circular

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCTTTTTTCT TTTCNNNNNC TTTTCTTTT TCTNNNNN  
38

20 (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: circular

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TTTCYTCGTT CGTCNNNNNC TACTTACTGC TTTNNNNN  
38

30 (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

35

-84-

1

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: circular

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCCTTCTTCY CCTCTNNNNN TCTCCGCTTC TTCCTNNNNN  
40

10 (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 34 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: both

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTTTTTCACA CTTTTTTTTT TTTCACACTT TTTT  
34

20 (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 34 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: both

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TCFTTCCACA CCTTCTTTT CTTCACACTT CTTT  
34

30 (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:

35

-85-

1

(A) LENGTH: 34 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: both

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TTTCTTCACA CTTCTTTTCT TTCCACACCT TTCT  
34

10 (2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AAAAAAAAAA AA  
12

20 (2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AAGAAAAGAA AG  
12

30 (2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

35

-86-

1

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CTTTCTTTTC TT  
12

10 (2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 12 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AAGAATAGAA AG  
12

20 (2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 12 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AAGAAUAGAA AG  
12

30 (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:

35

-87-

1

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CTTTCTATTC TT  
12

10 (2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: both

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TTCTTCTCTT TCCACACCTT TCTATTCTTC ACAC  
34

20 (2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AAAAAAAAAA AA  
12

30

35

-88-

1

WHAT IS CLAIMED:

1. A single-stranded circular oligonucleotide comprising at least one parallel binding (P) domain and at least one anti-parallel binding (AP) domain having a loop domain between each binding domain to form said circular oligonucleotide; each P and corresponding AP domain having sufficient complementarity to bind detectably to one strand of a defined nucleic acid target wherein said P domain binds in a parallel manner to said target, and said corresponding AP domain binds in an anti-parallel manner to said target.

2. The oligonucleotide of Claim 1 wherein said target comprises a known nucleotide sequence from which a nucleotide sequence for a sufficient number of positions in said P domain and in said corresponding AP domain is determined from the sequence of said target for said P domain:

when a base for a position in said target is guanine or a guanine analog, then P has cytosine, or a suitable analog thereof, in a corresponding position;

when a base for a position in said target is adenine, or an adenine analog then P has thymine or uracil, or suitable analogs thereof, in a corresponding position;

when a base for a position in said target is thymine, or a thymine analog, then P has cytosine or guanine, or suitable analogs thereof, in a corresponding position;

when a base for a position in said target is cytosine, or a cytosine analog, then P has cytosine,

35

-89-

1

thymine or uracil, or suitable analogs thereof, in a corresponding position; and

5 when a base for a position in said target is uracil, or a uracil analog, then P has cytosine, guanine, thymine or uracil, or suitable analogs thereof, in a corresponding position;

and for said AP domain:

10 when a base for a position in said target is guanine, or a guanine analog, then AP has cytosine or uracil, or suitable analogs thereof, in a corresponding position;

15 when a base for a position in said target is adenine, or an adenine analog, then AP has thymine or uracil, or suitable analogs thereof, in a corresponding position;

when a base for a position in said target is thymine, or a thymine analog, then AP has adenine, or a suitable analog thereof, in a corresponding position;

20 when a base for a position in said target is cytosine, or a cytosine analog, then AP has a guanine, or a suitable analog thereof, in corresponding position; and

when a base for a position in said target is uracil, or a uracil analog, then AP has adenine or  
25 guanine, or suitable analogs thereof, in a corresponding position;

wherein said sufficient number of positions is that number of positions to provide sufficient complementarity for said oligonucleotide to bind  
30 detectably to said target.

35



-90-

1

3. The oligonucleotide of Claim 1 wherein said  
P domain comprises a nucleotide sequence which is  
determined from a known nucleotide sequence of said  
5 target:

when a base for a position in said target is  
guanine or a guanine analog, then P has cytosine, or a  
suitable analog thereof, in a corresponding position;

10 when a base for a position in said target is  
adenine, or an adenine analog then P has thymine or  
uracil, or suitable analogs thereof, in a corresponding  
position;

when a base for a position in said target is  
thymine, or a thymine analog, then P has cytosine or  
15 guanine, or suitable analogs thereof, in a corresponding  
position;

when a base for a position in said target is  
cytosine, or a cytosine analog, then P has cytosine,  
thymine or uracil, or suitable analogs thereof, in a  
20 corresponding position;

when a base for a position in said target is  
uracil, or a uracil analog, then P has cytosine, guanine,  
thymine or uracil, or suitable analogs thereof, in a  
corresponding position;

25 and further wherein said AP domain comprises a  
nucleotide sequence which is determined from said  
sequence of said target as follows:

when a base for a position in said target is  
guanine, or a guanine analog, then AP has cytosine or  
30 uracil, or suitable analogs thereof, in a corresponding  
position;

35

-91-

1

when a base for a position in said target is adenine, or an adenine analog, then AP has thymine or uracil, or suitable analogs thereof, in a corresponding position;

5

when a base for a position in said target is thymine, or a thymine analog, then AP has adenine, or a suitable analog thereof, in a corresponding position;

when a base for a position in said target is cytosine, or a cytosine analog, then AP has a guanine, or a suitable analog thereof, in corresponding position; and

10

~~when a base for a position in said target is~~  
uracil, or a uracil analog, then AP has adenine or guanine, or suitable analogs thereof, in a corresponding position.

15

4. The oligonucleotide of Claim 1, 2 or 3 wherein said target, said P domain and said AP domain independently comprise from about 2 to about 200 nucleotides.

20

5. The oligonucleotide of Claim 4 wherein said target, said P domain and said AP domain independently comprise from about 6 to about 36 nucleotides.

6. The oligonucleotide of Claim 1, 2 or 3 wherein each loop domain independently comprises from about 2 to about 2000 nucleotides.

25

7. The oligonucleotide of Claim 6 wherein each loop domain independently comprises from about 3 to about 8 nucleotides.

8. The oligonucleotide of Claim 1, 2 or 3 wherein said target is single stranded or double stranded.

30

35

-92-

1

9. The oligonucleotide of Claim 1, 2 or 3 wherein said target is RNA or DNA.

5 10. The oligonucleotide of Claim 1, 2 or 3 wherein said target is a domain contained in a nucleic acid template.

11. The oligonucleotide of Claim 1, 2 or 3 wherein said P domain and said AP domain bind to said target in a staggered binding arrangement.

10 12. The oligonucleotide of Claim 1 or 2 wherein ~~sufficient complementarity is less than 100%~~ complementarity.

13. The oligonucleotide of Claim 12 wherein sufficient complementarity is about 30% to about 40%  
15 complementarity.

14. The oligonucleotide of Claim 1, 2 or 3 wherein said oligonucleotide is DNA or RNA.

15. The oligonucleotide of Claim 2 or 3 wherein a suitable analog of cytosine is 5-methylcytosine.  
20

16. The oligonucleotide of Claim 2 or 3 wherein a suitable analog of uracil is 5-methyluracil.

17. The oligonucleotide of Claim 2 or 3 ~~wherein a suitable analog of adenine is diaminopurine.~~

25 18. The oligonucleotide of Claim 1, 2 or 3 wherein nucleotides have a 2'-O-methylribose in place of ribose or deoxyribose.

19. The oligonucleotide of Claim 1, 2 or 3 wherein said oligonucleotide is taken up in a cell.

30 20. The oligonucleotide of Claim 19, wherein said oligonucleotide further comprises a ligand for a

35

-93-

1

cellular receptor, cholesterol group, an aryl group, a steroid group or a polycation.

5

21. The oligonucleotide of Claim 1, 2 or 3 wherein said oligonucleotide further comprises a drug or a drug analog.

10

22. The oligonucleotide of Claim 1, 2 or 3 wherein said loop domains comprise non-nucleotide loop domains.

23. The oligonucleotide of Claim 22 wherein said non-nucleotide loop domains are polyethylene glycol.

24. The oligonucleotide of Claim 23 wherein said polyethylene glycol is pentaethylene glycol, hexaethylene glycol or heptaethylene glycol.

15

25. The oligonucleotide of Claim 1, 2 or 3 wherein said oligonucleotide further comprises at least one methylphosphonate, phosphorothioate, phosphorodithioate, phosphotriester, siloxane, carbonate, acetamidate, thioether or phosphorus-boron linkage.

20

26. The oligonucleotide of Claim 1, 2 or 3 wherein said oligonucleotide further comprises a reporter molecule.

27. A compartmentalized kit for detection or diagnosis of a target nucleic acid, comprising:

25

~~at least one first container providing a circular oligonucleotide of any one of Claims 1-3.~~

30

28. A compartmentalized kit for isolation of a template nucleic acid, comprising at least one first container providing a circular oligonucleotide of Claim 1, 2 or 3, wherein said oligonucleotide is complementary to a target contained within said template.

35

-94-

1

29. The kit of Claim 28 wherein said template is poly (A)<sup>+</sup> mRNA.

5 30. A method of regulating biosynthesis of a DNA, an RNA or a protein which comprises:

contacting at least one oligonucleotide of any one of Claims 1 to 3 with a nucleic acid template for said DNA, said RNA or said protein, under conditions sufficient to permit binding of said at least one  
10 oligonucleotide to a target sequence contained within said template;

binding said oligonucleotide to said target;  
blocking access to or allowing degradation of said template and thereby regulating biosynthesis of said  
15 DNA, said RNA or said protein.

31. The method of Claim 30 wherein said template comprises a double-stranded nucleic acid target, and wherein said conditions are effective to denature said target by strand displacement and thereby permit  
20 binding.

32. The method of Claim 30 wherein said biosynthesis comprises at least one of DNA replication, DNA reverse transcription, RNA transcription, RNA splicing, RNA polyadenylation, RNA translocation and  
25 protein translation.

33. The method of Claim 32 wherein said template for said DNA replication is an RNA template or a DNA template.

34. The method of Claim 33 wherein said target  
30 of said oligonucleotide for regulating said DNA

35

-95-

1

replication is an origin of replication or a primer binding site.

5 35. The method of Claim 32 wherein said target of said oligonucleotide for regulating said DNA reverse transcription is a primer binding site, a site in a retroviral genome, or a site in an mRNA.

10 36. The method of Claim 32 wherein said target of said oligonucleotide for regulating said RNA transcription is a promoter, a repressor binding site, an operator, an enhancer, a transcription regulatory element or a site in an mRNA encoding region.

15 37. The method of Claim 32 wherein said target of said oligonucleotide for regulating said RNA splicing is at least one of a 5' splice junction, an intron branch point or a 3' splice junction.

20 38. The method of Claim 32 wherein said target of said oligonucleotide for regulating said RNA polyadenylation is a polyadenylation site.

39. The method of Claim 32 wherein said target of said oligonucleotide for regulating said RNA translocation is a poly(A) tail.

25 40. The method of Claim 32 wherein said template for said protein translation is an mRNA template.

41. The method of Claim 40 wherein said target of said template is a ribosome binding site, a 5' mRNA cap or a site in a protein coding region.

30 42. The method of Claim 30 wherein said template is a viral DNA or RNA template.

35

-96-

1

43. The method of Claim 42 wherein said oligonucleotide has a nucleotide sequence of SEQ ID NO: 3 or SEQ ID NO: 4.

5

44. A method of strand displacement in a double-stranded nucleic acid target which comprises contacting said target with a circular oligonucleotide of any one of Claims 1-3 for a time and under conditions effective to denature said target and to  
10 bind said circular oligonucleotide.

45. The method of Claim 44 wherein said conditions effective to denature said target comprise having said circular oligonucleotide and said target present in a ratio of about 1 to about 100.

15

46. The method of Claim 45 wherein said time effective to denature said target ranges from about 1 minute to about 16 hours.

47. The method of Claim 44 wherein said double-stranded nucleic acid target comprises a viral, a  
20 bacterial, a fungal or a mammalian nucleic acid.

48. The method of Claim 47 wherein said double-stranded nucleic acid target is an origin of replication, a promoter, a repressor binding site, an operator, an enhancer, a transcription regulatory element  
25 or a site in an mRNA encoding region.

49. The method of Claim 44 wherein said double-stranded nucleic acid target is present in a pure or impure nucleic acid sample, a tissue section, a cell smear or a chromosomal squash.

30

35

-97-

1

50. The method of Claim 49 wherein said oligonucleotide is covalently linked to a reporter molecule.

5

51. A pharmaceutical composition for regulating biosynthesis of a nucleic acid or protein comprising a biosynthesis regulating amount of at least one of the oligonucleotides of Claims 1 to 3 and a pharmaceutically acceptable carrier.

10

52. A method of preparing the single-stranded circular oligonucleotide of Claim 1, 2 or 3 comprising ~~binding a linear precircle to an end-joining-~~ oligonucleotide, joining two ends of said precircle and recovering said single-stranded circular oligonucleotide.

15

53. The method of Claim 52 wherein said linear precircle has a 3'-phosphate.

54. The method of Claim 53 wherein said two ends comprise two nucleotides corresponding to AP nucleotides of said single-stranded circular  
20 oligonucleotide.

55. The method of Claim 54 wherein said joining is performed with BrCN, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide or N-cyanoimidazole  $\text{ZnCl}_2$ .

25

~~56. A complex formed between the~~  
oligonucleotide of Claim 1, 2 or 3 and a target.

57. A method of specific cell type drug delivery comprising:

a) administering to an animal a drug  
30 covalently linked to an oligonucleotide of Claim 1, 2 or 3;

35



-98-

1

b) binding said oligonucleotide to a target mRNA present in said cell type; and

5 c) thereby delivering said drug to said specific cell type.

58. A method of detecting a target nucleic acid which comprises:

10 contacting a circular oligonucleotide of any one of Claims 1 to 3 with a sample to be tested for containing said nucleic acid for a time and under conditions sufficient to form an oligonucleotide-target complex; and

detecting said complex.

15 59. The method of Claim 58 wherein said nucleic acid comprises a double-stranded nucleic acid target, and wherein said conditions are effective to denature said target by strand displacement and thereby permit binding of said oligonucleotide to form said oligonucleotide-target complex.

20 60. The method of Claim 58 wherein said sample comprises a pure or impure nucleic acid sample, a tissue section, a cell smear or a chromosomal squash.

61. The method of Claim 58 wherein said conditions effective to denature said target comprise  
25 having said circular oligonucleotide and said target present in a ratio of about 1 to about 100.

62. The method of Claim 58 wherein said time effective to denature said target ranges from about 1 minute to about 16 hours.

30

35

-99-

1

63. The method of Claim 58 wherein said  
complex is detected by a fluorescence energy transfer  
assay.

5

64. The oligonucleotide of Claim 12 wherein  
sufficient complementarity is at least about 50%.

10

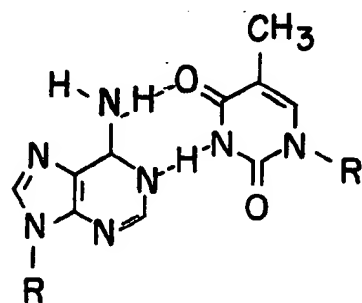
15

20

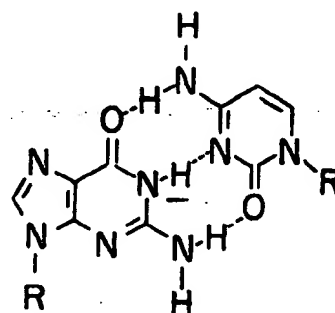
25

30

35

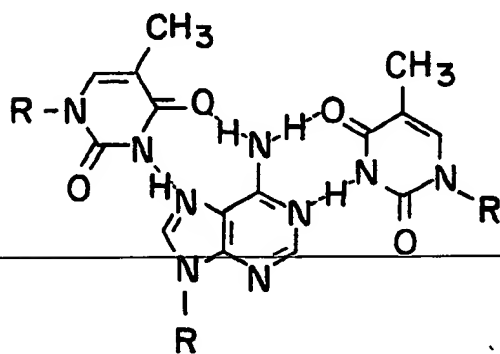
FIG. 1A 1 / 8

AT

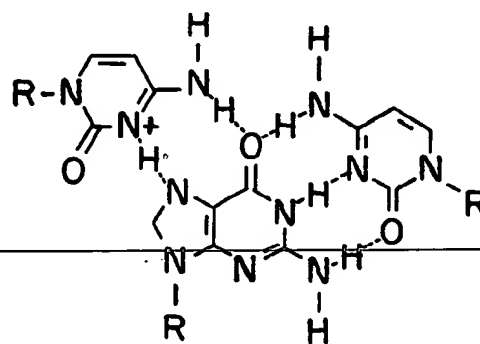


GC

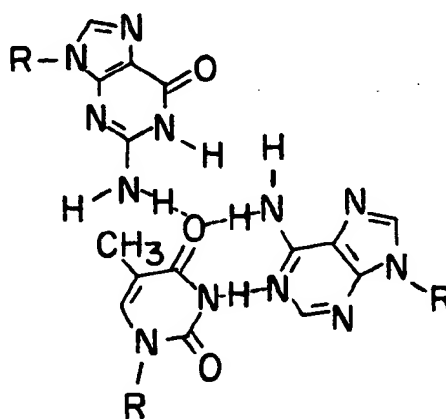
FIG. 1B



T-AT



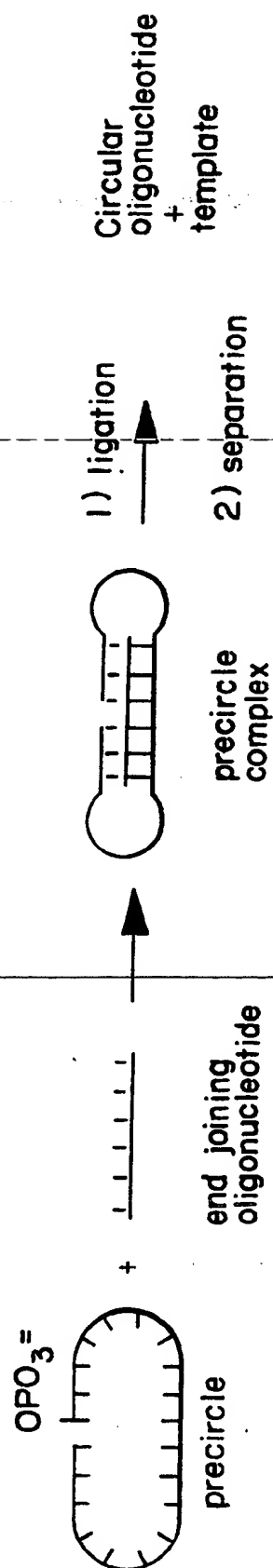
C+GC



G-TA

2 / 8

FIG. 2



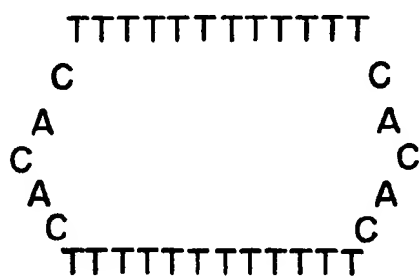
3 / 8

## FIG.3

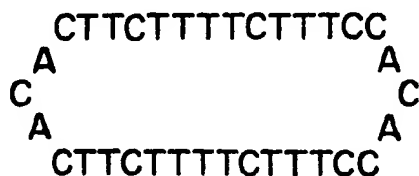
Precircles (1-3), Targets (4-5), Linear Oligonucleotides (9) and  
Circles (6-8) used in Experiments

- 1 5'-TTTTTTCACACTTTTTTTTTTTTTCACACTTTTTTT (SEQ ID NO: 5)  
 2 5'-TCTTTCCACACGTTTCTTTTCTTCACACTTCTTT (SEQ ID NO: 6)  
 3 5'-TTTCTTCACACTTCTTTTCTTTCCACACCTTTCT (SEQ ID NO: 7)  
 4 5'-AAAAAAAAAAAAA (SEQ ID NO: 8)  
 5 5'-AAGAAAAGAAAG (SEQ ID NO: 9)

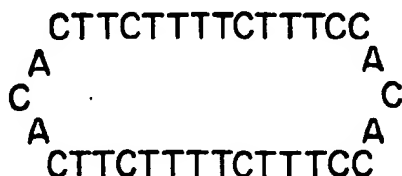
6 (SEQ ID NO: 5)



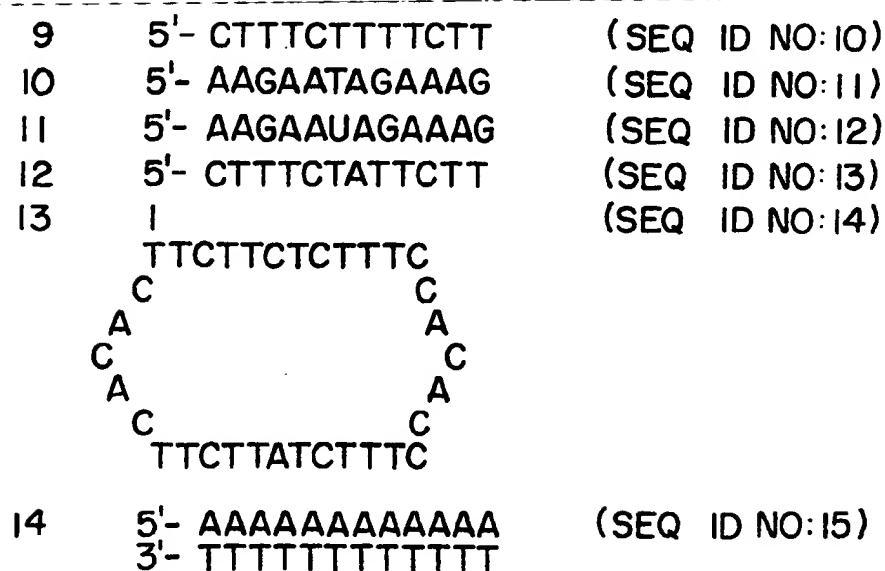
7 (SEQ ID NO: 6)



8 (SEQ ID NO: 7)



4 / 8

**FIG. 3**  
CONT.



6 / 8

FIG. 6B

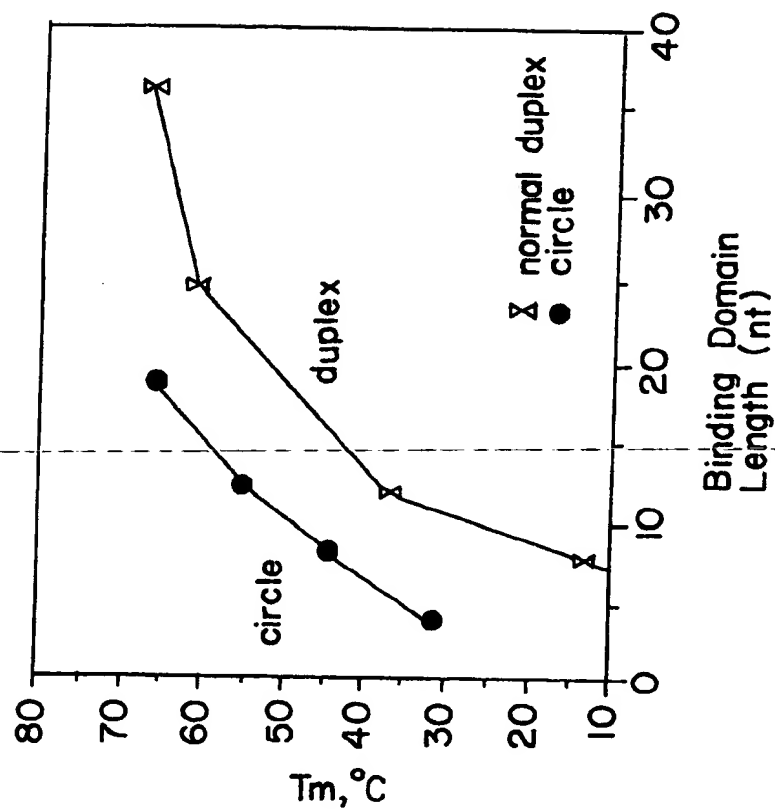
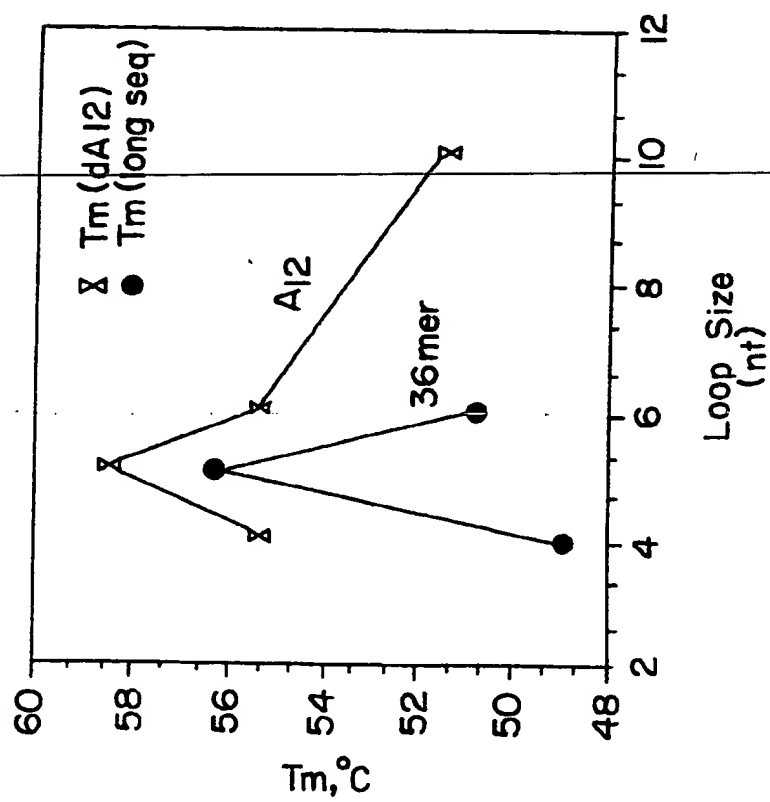


FIG. 6A

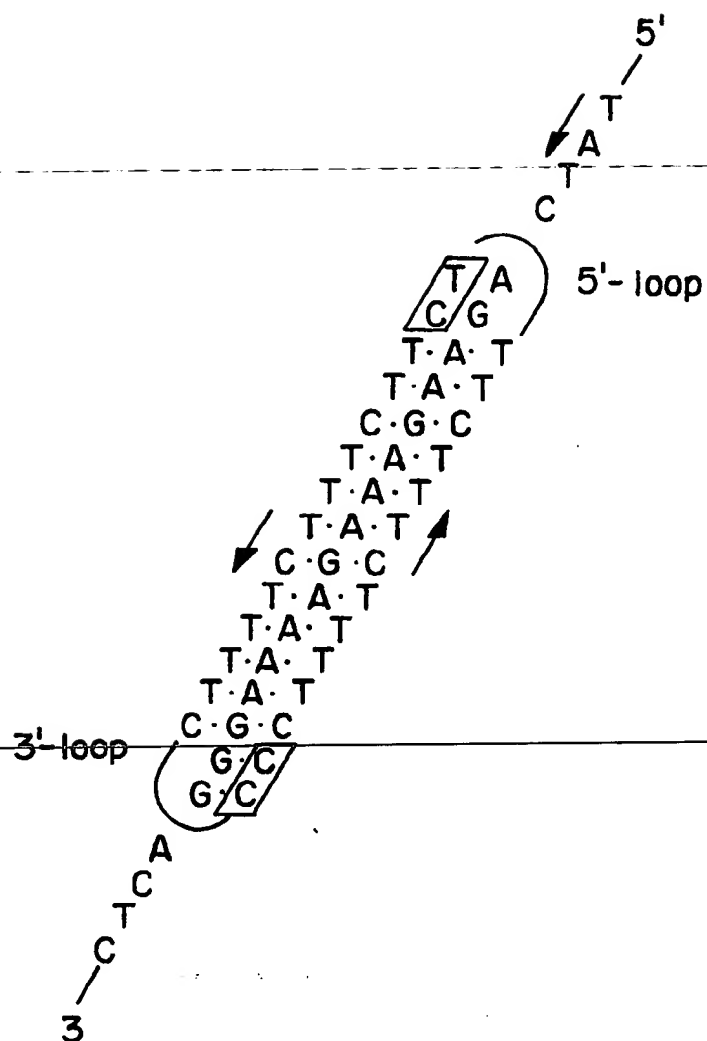


SUBSTITUTE SHEET



7 / 8

FIG. 7



8 / 8

FIG. 8

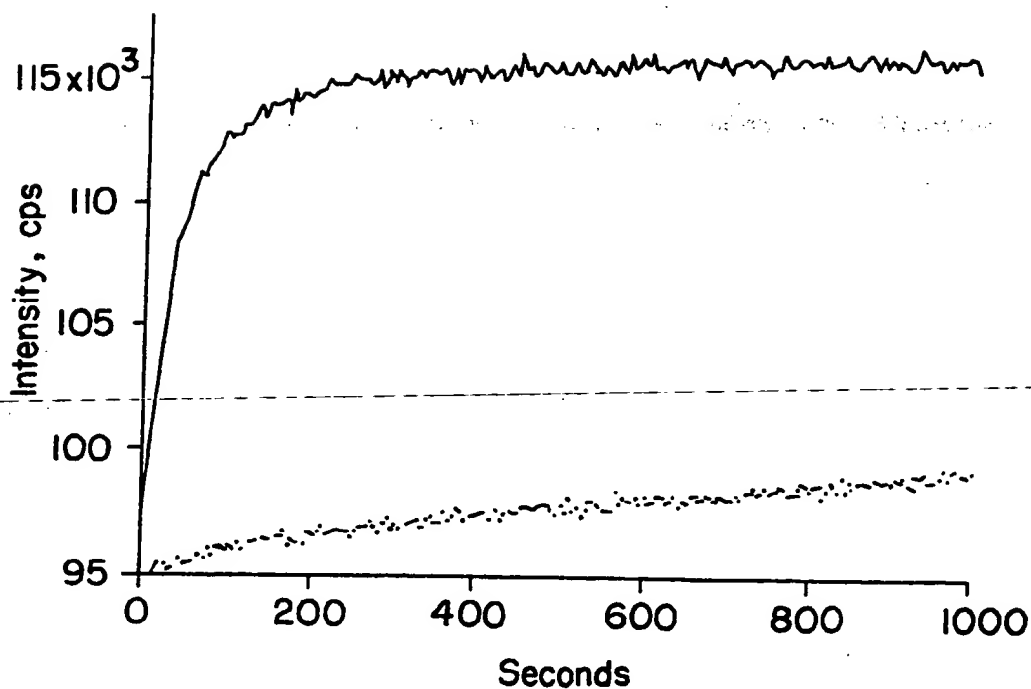
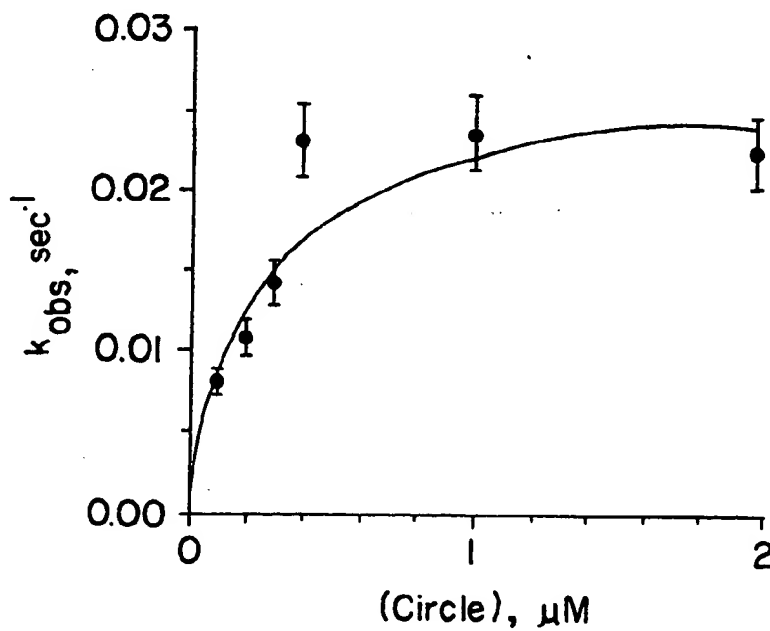


FIG. 9



SUBSTITUTE SHEET